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Chapter 7

IT IS MISLEADING TO USE *SAPAJUS* (ROBUST CAPUCHINS) AS A GENUS? A REVIEW OF THE EVOLUTION OF THE CAPUCHINS AND SUGGESTIONS ON THEIR SYSTEMATICS

Manuel Ruiz-García**, *María Ignacia Castillo and Kelly Luengas-Villamil

Laboratorio de Genética de Poblaciones-Biología Evolutiva, Unidad de Genética. Departamento de Biología. Facultad de Ciencias, Pontificia Universidad Javeriana. Bogotá DC., Colombia

ABSTRACT

The systematics and the evolutionary history of the capuchin monkeys is highly controversial. Recently, Lynch-Alfaro et al., (2012a, b) proposed to split the traditional *Cebus* genus into two genera, *Cebus* (gracile capuchins) and *Sapajus* (robust capuchins) and they also proposed a hypothesis to explain the evolution of these Neotropical primates ("Reinvasion of the Amazon"). Additionally, Bobli et al., (2012) suggested splitting the gracile capuchins into at least 12 species, although traditionally they had been classified into four species. Nevertheless the work of Lynch-Alfaro et al., (2012a) was criticized because of the small number of genes used and limited sample size (Nascimento et al., 2015). Good resolution of a species tree requires the correct identification of species, data from several loci, a high number of individuals per species, and careful analysis of ancient DNA data from museum specimens. Herein, we analyzed 452 capuchin monkeys (both gracile and robust groups) for four mitochondrial genes and

* Correspondence: mruizgar@yahoo.es, mruiz@javeriana.edu.co.

a subset of 27 individuals for 16 mitochondrial genes. There were four main findings: 1- Genetic distance values between *Cebus* and *Sapajus* were within the range of different species within a genus but significantly less than the values among different genera of Neotropical primates. 2- Genetic diversity was considerably higher in the gracile capuchins than in the robust capuchins. 3- Neither genetic tree showed the monophylia between *Cebus* and *Sapajus.* 4- The evolutionary history of the mitochondrial haplotypes within gracile capuchins and robust capuchins began around 4-5 and 3 million years ago (MYA) respectively.

Our results along with a review of karyological, morphological, and ethological data of capuchin monkeys do not support a split of the capuchins into two different genera. Also, our hypothesis, "North Amazon gracile origin, Eastern expansion and subsequent reinvasion of the Amazon," more completely explains the evolution of capuchins than does "Reinvasion of the Amazon" hypothesis of Lynch-Alfaro et al., (2012a). The Biological Species Concept (BSC) should be applied to the capuchins more than the Phylogenetic Species Concept (PSC), because we have enough data on many biological aspects of this group. The gracile capuchins should also be classified in a unique species, *C. capucinus* (including the traditional *C. capucinus, C. albifrons* and *C. olivaceus*) with different lineages intermixed in many geographical areas, whilst the robust capuchins should be classified into at least three species *C. xantosthernos, C. nigritus* and *C. apella.* It is difficult to apply traditional rules of systematic nomenclature to capuchins. Gracile capuchins underwent many migration events, had high gene flow and there is evidence of continuous mixture among different lineages. In contrast, robust capuchins had explosive Pleistocene radiation, colonized a wide array of extremely different biomes, and generated extreme diversity in morphology.

The situation of *C. apella* should be similar to that of our own species. Explosive radiation occurred in humans within the last 0.1-0.2 MYA allowing us to conquer extremely different ecological conditions, even using tools. Thus, from a phylogenetic point it's probably not important to focus on coat morphological differences for capuchins.

Keywords: *Cebus, Sapajus*, mitochondrial genes, evolution of capuchin monkeys, speciation, Biological Species Concept, Phylogenetic Species Concept

INTRODUCTION

The systematics and the reconstruction of the evolutionary history of the capuchin monkeys has been a central topic for many Neotropical primatologists. These monkeys have long captured our attention due to their creative and sophisticated behavior (including use of tools), their highly developed social trends and the wide array of different biomes that they colonized (Fragaszy et al., 2004).

Traditionally, two groups of these monkeys have been defined, the gracile (or untufted) and the robust (tufted) capuchins. Hershkovitz (1949) recognized three species of gracile capuchins, which have been widely accepted. They are the white-fronted capuchin, *Cebus albifrons* (Humboldt, 1812), the white-faced capuchin, *C. capucinus* (Linnaeus, 1758), and the wedge-capped or weeper capuchin, *C. olivaceus* (Schomburgk, 1848). More recently, a fourth species, the ka'apor capuchin, *C. kaapori*, was described by Queiroz (1992). Hershkovitz (1949) also defined 13 subspecies of *C. albifrons*, five of *C. capucinus* and five of *C. olivaceus*.

Here is a breakdown of the 13 subspecies of *C. albifrons.* 1- *C. a. albifrons* was identified by Humboldt (1812) in the Maipurés and Atures rapids along the border of the Orinoco River in Colombia and Venezuela. Defler and Hernández-Camacho (2002) determined that its Colombian distribution was in the lower Tuparro (left bank), Tuparrito, Tomo, Bita and Meta (right bank) rivers. 2- *C. a. hypoleucus* is within the Bolivar Department (Sinu River) in Northern Colombia. It was later assigned to *C. capucinus.* 3- *C. a. malitiosus* is distributed in the deciduous and humid forests of the northern and eastern slopes of the Santa Marta Sierra Nevada to elevations as high as 1300 m above sea level including Tayrona National Park in Colombia. 4- *C. a. cesarae* is distributed within the Magdalena Department south of Santa Marta Ciénaga Grande and in the lowlands of the Cesar Department from the vicinity of El Banco and Tamalameque northwards to the deciduous and gallery forests of the Rancheria River in the southern Guajira Department. Its geographical distribution encloses the Perijá Serrania, east of Valledupar, the Calancala and Ariguaní rivers and the east bank of the Magdalena River in Colombia. 5- *C. a. pleei* is distributed in swamps around Norosi, in the extreme north of Central Cordillera until Monpós, and along the west bank of the Magdalena River, in Colombia. 7- *C. a. versicolor* inhabits the Tolima Department and the middle Magdalena Valley (including the Monpós Island) in the Cesar Department. It is also distributed in the southeastern Sucre Department between the San Jorge and lower Cauca rivers, in the northern part of the Antioquia Department eastward from the Cauca River, including the Nechí River and in the eastern Boyacá Department as well as in the Caldas, Santander and the western Cundinamarca Departments in Colombia. 7- *C. a. leucocephalus* has a distribution range that extends from the eastern bank of the Magdalena River to the humid lowland Catatumbo region of the Norte de Santander Department in Colombia. 8- *C. a. adustus* was first found at the Cogollo River, five km northwest of Machiques, in the Zulia Department of Venezuela. It is distributed in the Maracaibo Lake region (Venezuela) and from the eastern base of the Sierra de Perija in Venezuela and Colombia. It's possible that this taxon is distributed across the piedmont forests of the western Arauca Department, at the northern tip of the Boyacá Department and in the eastern tip of the Norte de Santander Department. 9- *C. a. unicolor* was first observed near the mouth of the Tefé River in the Brazilian Amazon. It also, has a distribution range that includes the east bank of the Ucayali River and a large fraction of the Peruvian Amazon River, including the Alto Purús River. It also includes the Northern Bolivian Amazon. 10- *C. a. yuracus* was first described in Montalvo along the north bank of the Bobonaza River, 45 km from its confluence with the Pastaza River in Eastern Ecuador. In Colombia, this taxon is distributed in the southwestern Putumayo Department, south of the Guamués River. In the Peruvian Amazon, this taxon also inhabits the northern areas, including the Marañon and Napo Rivers as well as south of the Marañón River including the Ucayali River, and both banks of the Huallaga River until the Pachitea River, near Pucallpa. 11- *C. a. cuscinus* inhabits the southeastern Peruvian Amazon from the Alto Purús River up to Northern Bolivia as well as the lower parts of the Junin and Cuzco Peruvian Departments. This taxon seems to be frequently within the Manú National Park (upper Madre de Dios River, Peru; Terborgh, 1983). 12- *C. a. aequatorialis* is from the Pacific Coast in Western Ecuador and also in the Tumbes region in Northern Peru. 13- *C. a. trinitatis*, the last one, is from the Trinidad Island.

Hernández-Camacho and Cooper (1976) reinterpreted the arrangement of the Colombian forms proposed by Hershkovitz (1949). They included *leucocephalus* and *pleei* within *C. a. versicolor* because in 1958, the first author observed individuals representing the color-phases

of the three previously defined subspecies (dark phase – *C. a. leucocephalus*; light phase – *C. a. pleei* and intermediate phase – *C. a. versicolor*) in a single troop (in the region of Barrancabermeja at the eastern bank of the middle Magdalena River). Later, Groves (2001, 2005) further reduced the number of subspecies of *C. albifrons* to six, recognizing just one northern Colombian form, *C. a. versicolor* (*leucocephalus*, *malitiosus*, *adustus*, *cesarae*, and *pleei* as synonyms), and three Amazonian forms, *C. a. albifrons*, *C. a. cuscinus* (*yuracus* as a junior synonym), and *C. a. unicolor*, along with *C. a. trinitatis* from Trinidad, and *C. a. aequatorialis* from the Pacific coast in Ecuador and Peru. Defler and Hernández-Camacho (2002) confirmed, from a phenotypical perspective, that *C. a. albifrons* and *C. a. unicolor* were synonymous. They also stated that the Arauca Department populations originally classified as *C. a. adustus* and later enclosed by Groves (2001) inside *C. a. versicolor*, could also be enclosed within *C. a. albifrons*. However, Defler (2003; 2010) recognized *C. a. malitosus* and *C. a. cesarae* as two well-differentiated taxa from *C. a. versicolor*, disagreeing in this aspect from Groves (2001).

Table 1. Different morphological classifications for the robust capuchins obtained by diverse authors. The taxonomic review of Groves (2001, 2005) for the tufted capuchins is identical to that of Rylands et al., (2000). C = *Cebus*; S = *Sapajus*

Elliot (1913)	Cabrera and Yepes (1940)	Cabrera (1957)
C. apella	*C. xanthosternos*	*C. apella*
C. fatuellus	*C. paraguayanus*	*C. a. apella*
C. f. fatuellus	*C. libidinosus*	*C. a. libidinosus*
C. f. peruanus	*C. macrocephalus*	*C. a. macrocephalus*
C. macrocephalus	*C. frontatus*	*C. a. margaritae*
C. azarae	*C. nigritus*	*C. a. nigritus*
C. azarae	*C. vellerosus*	*C. a. pallidus*
C. azarae pallidus	*C. fatuellus*	*C. a. paraguayanus*
C. libidinosus	*C. apella*	*C. a. robustus*
C. frontatus		*C. a. vellerosus*
C. variegatus		*C. a. versutus*
C. versuta		*C. a. xanthosternos*
C. cirrifer		
C. crassiceps		
C. caliginosus		
C. vellerosus		
Hill (1960)	Groves (2001)	Silva Jr (2001)
C. apella	*C. apella*	*S. apella*
C. a. apella	*C. a. apella*	*S. macrocephalus*
C. a. margaritae	*C. a. fatuellus*	*S. libidinosus*
C. a. fatuellus	*C. a. macrocephalus*	*S. cay*
C. a. tocantinus	*C. a. peruanus*	*S. nigritus*
C. a. macrocephalus	*C. a. tocantinus*	*S. robustus*
C. a. magnus	*C. a. margaritae*	*S. xanthosternos*
C. a. juruanus	*C. libidinosus*	
C. a. maranonis	*C. l. libidinosus*	
C. a. peruanus	*C. l. pallidus*	
C. a. pallidus	*C. l. paraguayanus*	
C. a. cay	*C. l. juruanus*	
C. a. libidinosus	*C. nigritus*	

C. a. robustus	*C. n. nigritus*	
C. a. frontatus	*C. n. robustus*	
C. a. nigritus	*C. n. cucullatus*	
C. a. xanthosternos	*C. xanthosternos*	

Hershkovitz (1949) proposed five subspecies of *C. capucinus*. 1- *C. capucinus*, was originally found in Northern Colombia, probably near to Cartagena de Indias. 2- *C. c. curtus* was found in Gorgona Island within the Pacific area of Colombia. 3- *C. c. nigripectus* was first found in Las Pavas, Cauca Valley, between Cali and Buenaventura in Colombia. 4- *C. c. limitaneus* is from Belize, Honduras, Nicaragua and Guatemala and 5- *C. c. imitator* is from Panama, including the islands of Coiba, and Costa Rica. Nevertheless, Hershkovitz (1949) suggested that there were several subspecies in Colombia, "pending a thorough study of ample material." More recently, Rylands et al., (2000) considered four subspecies, *C. c. capucinus* in Colombia and Ecuador, *C. c. curtus* from the Gorgona Island, and *C. c. limitaneus* and *C. c. imitator* in Central America. However, Hernández-Camacho and Cooper, (1976) and Mittermeier and Combra-Filho, (1981), for example, did not recognize these subspecies because they found pelage characters too variable to permit recognition of any subspecies. Groves (2001) was of the same opinion and he considered this species monotypic. Similarly, neither Defler (2003, 2010) (in Colombia) nor Silva Jr (2001) recognized any *C. capucinus* subspecies.

In the case of *C. olivaceus* (also named *C. nigrivittatus*), Hershkovitz (1949) suggested the following five subspecies: 1- *C. o. nigrivittatus* from the upper Branco River, in the Northern Brazilian Amazon; 2- *C. o. olivaceus* from the southern foot of Monte Roraima, in the Northern Brazilian Amazon; 3- *C. o. castaneus* described from Cayenne, French Guiana; 4- *C. o. apiculatus* from Cuara River, Venezuela; and 5- *C. o. brunneus* from the Northwestern coast of Venezuela. Neither Silva Jr. (2001) nor Groves (2001, 2005) considered any of the subspecies to be valid.

The systematics of the robust capuchins have been at least as diverse as that of the gracile capuchins (see Table 1). For example, Groves (2001) used coat color variation and tuft shape to divide the robust capuchins into four species: *C. apella*, *C. libidinosus*, *C. nigritus*, *C. xanthosternos*: 1- *C. apella*, is the tufted or brown capuchin, and has six subspecies, *C. a. apella*, *C. a. fatuellus*, *C. a. macrocephalus*, *C. a. peruanus*, *C. a. tocantinus* and *C. a. margaritae* that are distributed in Venezuela, Surinam, Guianas, Brazil, Colombia, Ecuador, Peru and Bolivia. 2- *C. libidinosus*, is the bearded or black-striped capuchin that has four subspecies, *C. l. libidinosus*, *C. l. pallidus*, *C. l. paraguayanus* and *C. l. juruanus*. It is distributed in Brazil, Peru, Bolivia, Paraguay and Argentina. 3- *C. nigritus* is the black or black horned capuchin. It has three subspecies, *C. n. nigritus*, *C. n. robustus* and *C. n. cucullatus* and is distributed in Brazil, Argentina and Paraguay. 4- The fourth species is *C. xanthosternos*. It is the yellow-breasted or buff-headed capuchin and is endemic in the Atlantic Forest of Southern Bahia, north of the Jequitinhonha River. It is found at least as far north as the Paraguacú River, near Salvador in Brazil.

Silva Jr (2001) placed the robust capuchins into its own subgenus, *Sapajus* (Kerr, 1792), with seven species: 1- *C.* (*Sapajus*) *macrocephalus* and 2- *C. (Sapajus) apella* in the Amazon; 3- *C. (Sapajus) libidinosus* and 4- *C. (Sapajus) cay* in the Caatinga, Cerrado and Chaco habitats, and 5- *C. (Sapajus) robustus*, 6- *C. (Sapajus) nigritus* and 7- *C. (Sapajus) xanthosternos*, in the Atlantic Coastal Forest. The Marcgrave's capuchin, *Cebus flavius*, also

a robust capuchin, was recently rediscovered in Northeast Brazil (Mendes Pontes et al., 2006; Oliveira and Langguth, 2006).

Several studies have recently come forward to help clarify the systematics and the evolution of the capuchin monkeys. Here we summarize some of the most noteworthy molecular genetics studies:

1. Ruiz-García et al., (2010) were the first to analyze the intra-specific phylogeny of *C. albifrons* at the molecular level. They analyzed 118 specimens at the mitochondrial (mt) *COII* gene and showed the existence of three well defined groups in Northern Colombia: *malitosus*, *versicolor-pleei-cesarae* and *leucocephalus*. They arose from at least, three distinct migrations from different Amazonian groups. Five different Amazonian and Eastern Llanos *C. albifrons's* groups (I, II, III, IV, and V) were also found. Many of these groups did not correspond with the traditional morphological subspecies previously described. In many Amazonian localities, some of these groups live in sympatry probably by secondary expansion after their respective formations.
2. In a second study, focusing on the mt *Cyt-b* gene, Casado et al., (2010) estimated the molecular divergence of two separate populations of *C. cay*, and estimated its time of separation from *C. apella*. Twenty-three *C. cay* individuals from Brazil and nine from Paraguay showed 24 haplotypes (20 and 4, respectively), accounting for 29 variable sites (19 transitions and 10 transversions). The genetic distance between haplotypes averaged 0.5%, with 1.1% between *C. cay* populations. Mismatch distribution indicated that this species suffered a recent demographic expansion. Divergence time estimates suggested that the two populations of *C. cay* split in the Pleistocene.
3. Ruiz-García et al., (2012a) were the first to analyze, molecularly, the intra-specific phylogeny of *C. capucinus* (121 individuals analyzed). Four different and significant haplotype lineages were found in Colombia living sympatrically in the same Departments of this country. They all presented high levels of gene diversity but the III Colombian mitochondrial haplogroup was determined likely to be the most ancestral lineage. The II Colombian mitochondrial haplogroup was probably the source of origin of the unique Central America mitochondrial haplogroup that was detected. These molecular population genetics data do not agree with the existence of two well-defined subspecies in Central America (*limitaneus* and *imitator*). This Central America mitochondrial haplogroup showed significantly less genetic diversity than the Colombian mitochondrial haplogroups. All the *C. capucinus* analyzed showed evidence of historical population expansions.
4. Lynch-Alfaro et al., (2012a), analyzed 53 specimens of capuchin monkeys (eight *C. capucinus*, 12 *C. olivaceus*, 13 *C. albifrons*, six *S. microcephalus*, two *S. apella*, three *S. cay*, two *S. libidinosus*, two *S. xanthosternos* and five *S. nigritus*) at two mt genes (*12S rRNA* and *Cyt-b*), and determined that the capuchin monkeys contained two well supported monophyletic clades, the morphologically distinct gracile and robust groups. They considered these groups to be two well-separated genera of capuchins (*Cebus* and *Sapajus*). They estimated a late Miocene divergence between *Cebus* and *Sapajus* and a subsequent Plio-Pleistocene diversification within each of the two clades. A Bayesian analysis indicated that the current wide-ranging sympatry of *Cebus* and *Sapajus* across much of the Amazon Basin was the result of a single

explosive late Pleistocene invasion of *Sapajus* from the Atlantic Forest into the Amazon, where *Sapajus* is now sympatric with gracile capuchins across much of their range. They considered three different hypotheses to explain the distribution of capuchin monkeys and the sympatry of both robust and gracile groups in the Amazon. The first hypothesis, "Out of the Amazon," explains the sympatry of robust and gracile capuchins because the Amazon is the origin focus for all capuchin monkeys. They evolved by allopatric or sympatric speciation within the Amazon Basin. The gracile capuchins subsequently radiated north and the robust capuchins radiated south. This hypothesis agrees quite well with three assumptions. First, the Amazon Basin is the ancestral origin of the capuchin monkeys. Second, the diversification into gracile and robust capuchins also occurred within the Amazon. Third, a recent invasion of these two capuchin forms occurred towards northern South America, Central America, the Cerrado and the Atlantic Forest. This hypothesis predicts that the basal division for capuchins was north (gracile forms) and south (robust forms) of the Amazon River. The second hypothesis is the "Atlantic versus Amazon." This hypothesis is based in the vicariance between the Atlantic coastal forest and the Amazon Basin, the major force shaping capuchin monkey distributions. Different examples with Neotropical primates (*Callithrix* vs. *Mico* + *Cebuella*; Tagliaro et al., 1997) and many other vertebrates (with divergence between Atlantic coastal forest and the Amazon) can be related (Patton et al., 2000). This hypothesis predicted that the robust capuchins would be the ancestral condition, and the gracile capuchins would have evolved relatively recently from a robust ancestor in the Amazon. Three predictions emerge from this hypothesis. First, there is a basal split within capuchins between robust Atlantic and robust Amazon taxa. Second, there is paraphyly of robust capuchins, with the robust Atlantic clade as the sister group to the Amazon gracile capuchins + Amazon robust capuchins. This means that gracile capuchins are a subclade of the Amazon robust capuchins). Third, there is a relatively recent origin of gracile capuchins. There is a third hypothesis: "Reinvasion of the Amazon." This hypothesis affirms that after a basal gracile Amazon–robust Atlantic Forest divergence, the robust Atlantic Forest capuchin evolved in allopatry from the Amazon gracile capuchin, and later re-established sympatry when the robust form expanded across the Cerrado and into the Amazon. Here are some predictions of this hypothesis. First, there is initial divergence of a robust Atlantic capuchin clade and a gracile Amazon capuchin clade. Second, the robust Amazon capuchins forming a recently evolved subclade are nested with the Atlantic Forest robust clade.

Lynch-Alfaro et al., (2012a) performed two tests of the dispersal rate to evaluate these three hypotheses. If the "out of the Amazon" hypothesis is true, the prediction is that both robust and gracile capuchins began diversifying at the same time, and that each dispersed at relatively equal rates. Following this hypothesis, the Atlantic Forest robust capuchins, as the most distant dispersal group from the Amazon, might be expected to have the highest dispersal rate. If the "Atlantic versus Amazon" hypothesis is true, then, they predicted that the robust capuchins have been diversifying longer in both the Amazon and the Atlantic forests, which could explain their large distribution without recourse to rapid dispersal. Furthermore, the gracile capuchins, as a recently evolved subclade of the Amazonian robust capuchins, would

have rapidly colonized the areas where they currently live. This predicts a more rapid rate of dispersal for gracile compared with robust capuchins. If the "reinvasion of the Amazon" hypothesis is true, the gracile Amazon and the robust Atlantic Forest capuchins have been diversifying and dispersing the longest amount of time. But the relatively recent reinvasion and dispersal of robust capuchins throughout the Cerrado and the Amazon encompasses the largest area. This requires a more rapid dispersal rate for robust Amazon capuchins than that for either the gracile Amazon or the Atlantic Forest robust capuchins. They concluded that the "reinvasion of the Amazon" hypothesis was the best fit.

5. Lynch-Alfaro et al., (2012b) reviewed extensive morphological, genetic, behavioral, ecological, and biogeographic evidence and stated that there was sufficient data to split the capuchin monkeys into two full genera (*Cebus* and *Sapajus*).
6. Bobli et al., (2012) analyzed two mt genes (*Cyt-b* and *D-loop*) from 50 gracile capuchin samples. Their data indicate that the gracile capuchins underwent a radiation about 2 MYA ago and quickly diversified in both the Andes and the Amazon. These authors used an extreme typological PSC and split the gracile capuchin taxa into many species. They suggested at least two Amazonian species (*C. yuracus* and *C. unicolor*), a species from the Guiana Shield (*C. albifrons*), two Northern Andean species (*C. versicolor* and *C. cesarae*), *C. brunneus* on the Venezuelan coast, *C. adustus* in the region of Lake Maracaibo, *C. capucinus* in Northwestern Ecuador, Colombia and Panama, *C. imitator* in Central America, *C. olivaceus* and *C. castaneus* occupying a large part of the Guiana Shield and *C. kaapori* in the Eastern Amazon, south of the Amazon River.
7. Ruiz-García et al., (2012b) analyzed 49 robust capuchins that had exact geographic origins from diverse areas of Colombia, Peru, Bolivia, French Guiana, Brazil, Argentina and Paraguay. They determined different findings. First, they found two established and related taxa in the northern Amazon River area. They named *C. a. apella* and *C. a. fatuellus. C. a. apella* is distributed from French Guiana until, at least, the Negro River in the northern Brazilian Amazon. *C. a. fatuellus* is distributed throughout the Colombian Eastern Llanos and the northern Colombian Amazon. Second, they determined two other *C. apella* taxa in the southern Amazon area: *C. a. macrocephalus* and *C. a. cay*. *C. a. macrocephalus* has a western and southern Amazon distribution, while *C. a. cay* has a more southern distribution outside the Amazon Basin. In the upper and western Amazon Basin, there was a unique lineage (*C. a. macrocephalus*) with one widely distributed haplotype. Therefore, the four morphological subspecies or species described for this area (*C. a. maranonis, C. a. macrocephalus, C. a. peruanus, C. l. pallidus)* and maybe a fifth unknown subspecies were molecularly undifferentiated at least for the mitochondrial gene analyzed. They were all identified as *C. a. macrocephalus*. Third, the specimens classified as *C. nigritus* and *C. xanthosternos* were clearly differentiated from the other specimens. These two lineages were assigned to the status of full species.
8. Nascimento et al., (2015) criticized the work of Lynch-Alfaro et al., (2012a). They re-analyzed the data of these authors, including additional mt *Cyt-b* data from *S. xanthosternos* and *S. flavius*. They placed *S. xanthosternos* in a monophyletic clade representing the most basal lineage of the robust capuchins (this had already been

demonstrated by Ruiz-García et al., 2012b). Their analyses indicated polyphyletic arrangements for several capuchin species (a fact observed in the previously cited works). They concluded that the molecular data available at that moment lacked the adequate variation for accurately resolving species relationships. A better resolution of the species tree is required along with the correct identification of species, data from several unlinked nuclear loci from a higher number of individuals per species, and careful analysis of ancient DNA data from museum specimens.

In the current work we employed the nomenclature proposed by Lynch-Alfaro et al., (2012a, b) with *Cebus* and *Sapajus* as separated genera. However, pending our results we will either maintain or change this nomenclature.

Many of the most modern systematic changes in capuchin monkeys, as well as in many other organisms, vary depending on the species criteria used. For example, the BSC is arguably the strongest concept of species for sexually reproducing taxa (Mayr, 1942, 1963). This concept holds that species are sets of interbreeding populations that are reproductively isolated (with no fertile hybrids) from other similar sets. This means that this concept has taken into account the existence of pre-zygote isolation reproductive processes, which is a paradigm within the Neodarwinism synthesis (Barton and Bengtsson, 1986; Coyne et al., 1994; Antonovics, 2006). The BSC has two major operational problems. First, it is difficult to evaluate among populations in extreme allopatry. Second, the researchers must demonstrate if there are fertile hybrids among different taxa in the wild.

Other alternative species definitions are the genetic species concept (GSC; Baker and Bradley, 2006) and the PSC (Cracraft, 1983). The first one defines a species as a group of populations that are genetically isolated from other groups (two different genetic pools with independent evolutionary fates). Thus, this definition does not necessarily imply pre-zygote isolation reproductive processes. The second one defines a species as the smallest monophyletic and diagnosable cluster of individuals with a parental pattern of ancestry and descent by means of molecular or morphological characteristics. Operationally, this concept is easier to apply than the BSC, because a researcher could define new species without having to demonstrate if there are pre and/or post-zygote isolation reproductive mechanisms, strong karyotype differences, "normal" hybrids in the wild, etc. It's clear that if the researcher has reproduction data for a determined taxon or for a taxa set, the BSC is a more complete species concept than the PSC.

To help to clarify many of the controversy about systematics and the evolutionary history of the capuchin monkeys, we analyzed a set of 452 individuals (using the previous nomenclature: 124 *C. capucinus*, 240 *C. albifrons*, one *C. olivaceus*, two *S. xanthosternos*, two *S. nigritus*, four *S. cay*, 22 *S. a. macrocephalus*, one *S. robustus*, five *S. a. apella* and 51 *S. a. fatuellus*) sequenced for four mt genes (*D-loop*, *Cyt-b*, *COI* and *COII*). We included a greater number of specimens, geographic localities and genes compared to any previous capuchin study. Furthermore, a sub-set of these individuals (27 specimens: 14 *C. albifrons*, two *S. a. cay*, seven *S. a. macrocephalus*, one *S. a. apella* and three *S. a. fatuellus*) were sequenced for 16 mitochondrial genes.

Our study has four main aims: 1- To determine if the genetic distances of neutral or quasi neutral molecular markers among different taxa of gracile and robust capuchins are within the range of values among species of the same genus or within values among different Neotropical primate genera; 2- To analyze if the alleged monophylia between *Cebus* and

Sapajus claimed by Lynch-Alfaro et al., (2012a) is maintained when the sample size, number of geographical sights sampled, and the number of molecular markers are increased; 3- To determine if the "reinvasion of the Amazon" hypothesis and the temporal splits found by Lynch-Alfaro et al., (2012a) are maintained when the sample size, geographical localities and number of genes are increased; 4- To analyze if there is a correlation between the traditional morphological species and subspecies of capuchin monkeys and the results using molecular genetics data; and 5- If there is no correlation, to propose a new systematics for the capuchin monkeys.

MATERIALS AND METHODS

It's important to know the exact geographical origins of the individuals analyzed in order to resolve the evolutionary history of the capuchins (or, other taxa), (Nascimento et al., 2015). Indeed, sometimes it is easy to observe mistakes concerning the origin and distributions of some samples. For instance, Figure 1 in Lynch-Alfaro et al., (2012a) showed incorrect distributions of robust capuchins. Similarly, Nascimento et al., (2015) criticized the same study for erroneously classified some specimens (one from Ayacucho, two from the Loreto Department in the Peruvian Amazon, and two from the Meta Department in Colombia) as *macrocephalus.* In disagreement, Nascimento et al., (2015) concluded that they are *apella*. However, we have directly sampled many capuchins in these areas of Peru and Colombia. Based on our data, the Peruvian specimens are *macrocephalus*, agreeing with Lynch-Alfaro et al., (2012a). Again based on our data, neither work correctly classified the two Colombian individuals. They are *fatuellus*.

For this reason, we have directly sampled a total of 452 wild capuchin monkeys and documented their geographical origins.

The DNA was obtained from samples of hair, teeth, muscle and blood from animals found alive or dead in diverse Indian communities. We requested permission to collect biological materials from either carcasses or live animals that were already present in the community. We sampled small pieces of tissue (muscle or blood) or teeth from hunted animals that were discarded during the cooking process, or hairs with bulbs plucked from live pets. Communities were visited only once, all sample donations were voluntary, and no financial or other inducement was offered for supplying specimens for analysis. All the pets and the hunted animals analyzed were obtained by the Indian communities at a maximum of 15 km from the community.

These 452 individuals were from the following taxa. There were 124 *C. capucinus* (58 from Colombia representing the four haplogroups detected by Ruiz-García et al., 2012a, and 66 from diverse countries of Central America: Panama, Costa Rica and Guatemala). Another 240 were *C. albifrons*. This included 78 *C. a. versicolor*, 14 *C. a. pleei*, eight *C. a. cesarae*, five *C. a. adustus*, 34 *C. a. leucocephalus*, one *C. a. malitosus*, 47 *C. a. albifrons* (including 40 *C. a. unicolor* if we follow the nomenclature of Hershkovitz, 1949), 45 *C. a. yuracus*, three *C. a. cuscinus*, two *C. a. aequatorialis*, one individual from a undescribed subspecies from the Peruvian San Martin Department and two natural hybrids between *C. albifrons* and *C. capucinus*. All of these animals were sampled in Colombia, Ecuador, Peru, Bolivia and Brazil. Also there were: one *C. olivaceus castaneus* (French Guiana), two *S. xanthosternos*

(Bahia, Brazil), two *S. nigritus* (Misiones, Argentina), four *S. cay* (South Brazil and Paraguay), one *S. robustus* (Espirito Santo, Brazil), 22 *S. apella macrocrocephalus* (frontier between Argentina and Bolivia, Bolivia, Brazil, Peru and Colombia), five *S. apella apella* (French Guiana and Brazil) and 51 *S. apella fatuellus* (Colombia). We used a sample of *Aotus azarae boliviensis* (Santa Cruz Department, Bolivia) as an outgroup. All samples were completely sequenced for four mitochondrial genes. A subset of these capuchin monkeys were sequenced for 16 mitochondrial genes. The subset included 27 capuchin monkeys (six *C. a. versicolor*, one *C. a. pleei*, one *C. a. cesarae*, one *C. a. malitosus*, three *C. a. albifrons/unicolor*, one *C. a. yuracus*, one *C. a. aequatorialis*, three *S. apella fatuellus*, one *S. apella apella*, two *S. cay* and seven *S. apella macrocephalus*). It also contained 19 *Ateles geoffroyi* (Costa Rica), 10 *Lagothrix lagotricha cana* (Madeira River, Brazil), 10 *Alouatta palliata* (Costa Rica), 17 *Saimiri cassiquiarensis albigena* (Colombia), two *Aotus nancymaee* (Peru), seven *Aotus vociferans* (Colombia), one *Saguinus labiatus* (Peru), one *Saguinus fuscicollis* (Peru), 20 *Saguinus leucopus* (Colombia) and three *Saguinus oedipus* (Colombia).

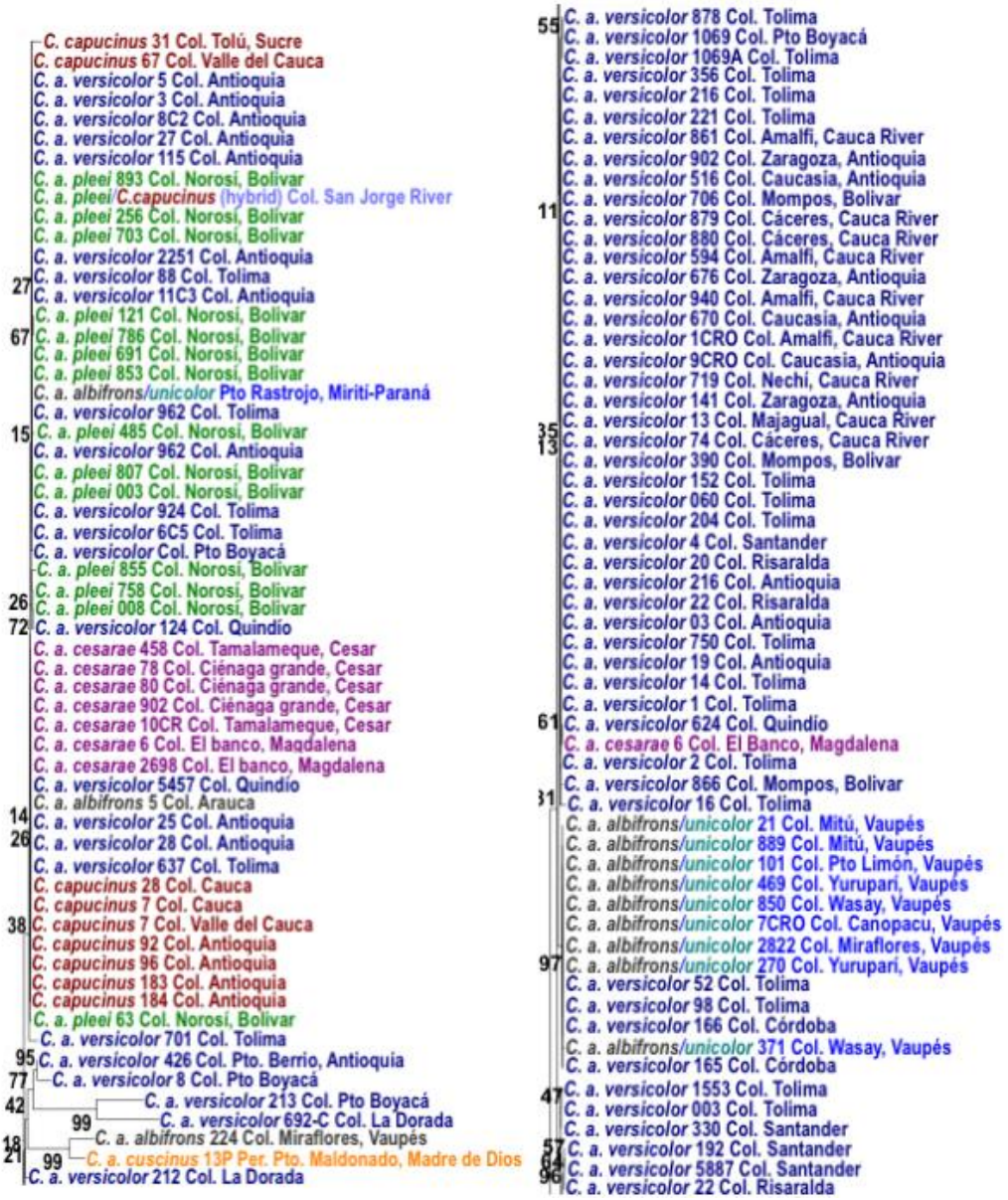
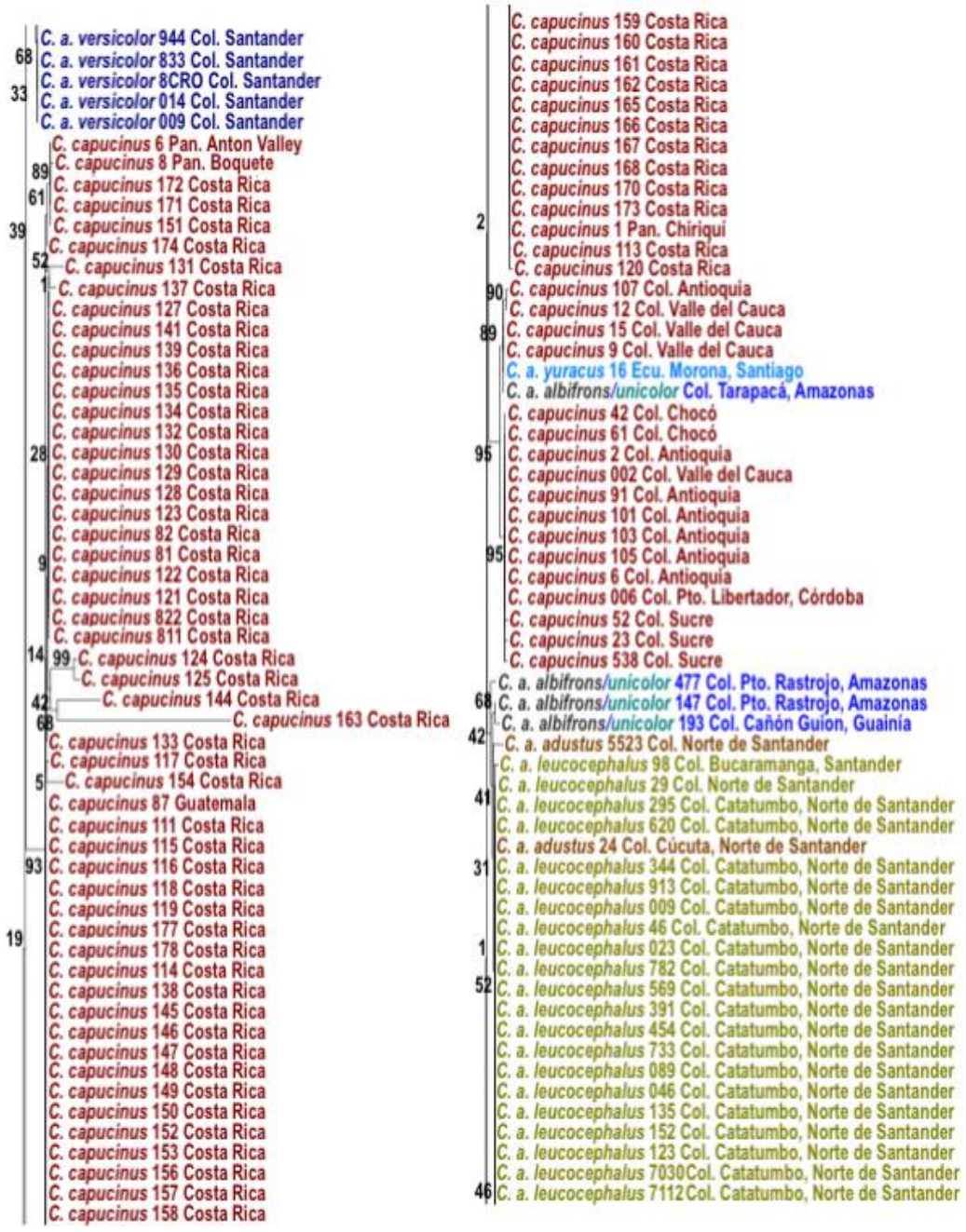


Figure 1. (Continued).


C. a. versicolor 944 Col. Santander
68 C. a. versicolor 833 Col. Santander
C. a. versicolor 8CRO Col. Santander
33 C. a. versicolor 014 Col. Santander
C. a. versicolor 009 Col. Santander
C. capucinus 6 Pan. Anton Valley
89 C. capucinus 8 Pan. Boquete
C. capucinus 172 Costa Rica
61 C. capucinus 171 Costa Rica
39 C. capucinus 151 Costa Rica
C. capucinus 174 Costa Rica
52 C. capucinus 131 Costa Rica
1 C. capucinus 137 Costa Rica
C. capucinus 127 Costa Rica
C. capucinus 141 Costa Rica
C. capucinus 139 Costa Rica
C. capucinus 136 Costa Rica
C. capucinus 135 Costa Rica
C. capucinus 134 Costa Rica
C. capucinus 132 Costa Rica
28 C. capucinus 130 Costa Rica
C. capucinus 129 Costa Rica
C. capucinus 128 Costa Rica
C. capucinus 123 Costa Rica
C. capucinus 82 Costa Rica
9 C. capucinus 81 Costa Rica
C. capucinus 122 Costa Rica
C. capucinus 121 Costa Rica
C. capucinus 822 Costa Rica
C. capucinus 811 Costa Rica
14 99 C. capucinus 124 Costa Rica
C. capucinus 125 Costa Rica
42 C. capucinus 144 Costa Rica
68 C. capucinus 163 Costa Rica
C. capucinus 133 Costa Rica
C. capucinus 117 Costa Rica
5 C. capucinus 154 Costa Rica
C. capucinus 87 Guatemala
C. capucinus 111 Costa Rica
C. capucinus 115 Costa Rica
93 C. capucinus 116 Costa Rica
C. capucinus 118 Costa Rica
C. capucinus 119 Costa Rica
19 C. capucinus 177 Costa Rica
C. capucinus 178 Costa Rica
C. capucinus 114 Costa Rica
C. capucinus 138 Costa Rica
C. capucinus 145 Costa Rica
C. capucinus 146 Costa Rica
C. capucinus 147 Costa Rica
C. capucinus 148 Costa Rica
C. capucinus 149 Costa Rica
C. capucinus 150 Costa Rica
C. capucinus 152 Costa Rica
C. capucinus 153 Costa Rica
C. capucinus 156 Costa Rica
C. capucinus 157 Costa Rica
C. capucinus 158 Costa Rica
C. capucinus 159 Costa Rica
C. capucinus 160 Costa Rica
C. capucinus 161 Costa Rica
C. capucinus 162 Costa Rica
C. capucinus 165 Costa Rica
C. capucinus 166 Costa Rica
C. capucinus 167 Costa Rica
C. capucinus 168 Costa Rica
C. capucinus 170 Costa Rica
C. capucinus 173 Costa Rica
2 C. capucinus 1 Pan. Chiriquí
C. capucinus 113 Costa Rica
C. capucinus 120 Costa Rica
90 C. capucinus 107 Col. Antioquia
C. capucinus 12 Col. Valle del Cauca
89 C. capucinus 15 Col. Valle del Cauca
C. capucinus 9 Col. Valle del Cauca
C. a. yuracus 16 Ecu. Morona, Santiago
C. a. albifrons/unicolor Col. Tarapacá, Amazonas
C. capucinus 42 Col. Chocó
C. capucinus 61 Col. Chocó
95 C. capucinus 2 Col. Antioquia
C. capucinus 002 Col. Valle del Cauca
C. capucinus 91 Col. Antioquia
C. capucinus 101 Col. Antioquia
C. capucinus 103 Col. Antioquia
95 C. capucinus 105 Col. Antioquia
C. capucinus 6 Col. Antioquia
C. capucinus 006 Col. Pto. Libertador, Córdoba
C. capucinus 52 Col. Sucre
C. capucinus 23 Col. Sucre
C. capucinus 538 Col. Sucre
C. a. albifrons/unicolor 477 Col. Pto. Rastrojo, Amazonas
68 C. a. albifrons/unicolor 147 Col. Pto. Rastrojo, Amazonas
C. a. albifrons/unicolor 193 Col. Cañón Guion, Guainía
42 C. a. adustus 5523 Col. Norte de Santander
C. a. leucocephalus 98 Col. Bucaramanga, Santander
C. a. leucocephalus 29 Col. Norte de Santander
41 C. a. leucocephalus 295 Col. Catatumbo, Norte de Santander
C. a. leucocephalus 620 Col. Catatumbo, Norte de Santander
C. a. adustus 24 Col. Cúcuta, Norte de Santander
31 C. a. leucocephalus 344 Col. Catatumbo, Norte de Santander
C. a. leucocephalus 913 Col. Catatumbo, Norte de Santander
C. a. leucocephalus 009 Col. Catatumbo, Norte de Santander
C. a. leucocephalus 46 Col. Catatumbo, Norte de Santander
1 C. a. leucocephalus 023 Col. Catatumbo, Norte de Santander
C. a. leucocephalus 782 Col. Catatumbo, Norte de Santander
52 C. a. leucocephalus 569 Col. Catatumbo, Norte de Santander
C. a. leucocephalus 391 Col. Catatumbo, Norte de Santander
C. a. leucocephalus 454 Col. Catatumbo, Norte de Santander
C. a. leucocephalus 733 Col. Catatumbo, Norte de Santander
C. a. leucocephalus 089 Col. Catatumbo, Norte de Santander
C. a. leucocephalus 046 Col. Catatumbo, Norte de Santander
C. a. leucocephalus 135 Col. Catatumbo, Norte de Santander
C. a. leucocephalus 152 Col. Catatumbo, Norte de Santander
C. a. leucocephalus 123 Col. Catatumbo, Norte de Santander
C. a. leucocephalus 7030Col. Catatumbo, Norte de Santander
46 C. a. leucocephalus 7112Col. Catatumbo, Norte de Santander


C. a. leucocephalus 124 Col. Catatumbo, Norte de Santander
C. a. leucocephalus 853 Col. Catatumbo, Norte de Santander
C. a. leucocephalus 023 Col. Catatumbo, Norte de Santander
C. a. leucocephalus 3 Col. Catatumbo, Norte de Santander
C. a. leucocephalus 7 Col. Moniquirá, Boyacá
C. a. adustus 8 Col. Arauca
C. a. leucocephalus 206 Col. Norte de Santander
C. a. leucocephalus 233 Col. Norte de Santander
C. a. leucocephalus 234 Col. Norte de Santander
C. a. leucocephalus 117 Col. Norte de Santander
C. a. adustus 1524 Col. Norte de Santander
C. a. adustus 4 Col. Norte de Santander
C. capucinus 68 Col. Buenavista, Valle del Cauca
C. capucinus 69 Col. Buenavista, Valle del Cauca
C. a. leucocephalus 690 Col. Buenavista, Valle del Cauca
C. a. leucocephalus 187 Col. Buenavista, Valle del Cauca
C. a. leucocephalus 136 Col. Buenavista, Valle del Cauca
C. a. leucocephalus 204 Col. Buenavista, Valle del Cauca
C. cuscinus 252 Bol. Ribera alta, Beni
C. cuscinus 253 Bol. Ribera alta, Beni
C. a. yuracus 202 Ecu. Pastaza
C. a. yuracus 108 Per. Ucayali River
C. a. albifrons/unicolor 281 Bra. Tefé Lake, Amazonas
C. a. albifrons/unicolor 107 Bra. Negro River
C. a. albifrons/unicolor 90 Bra. Tefé Lake, Amazonas
C. a. albifrons/unicolor 246 Bra. Santa Sofia, Amazonas
C. a. albifrons/unicolor 247 Bra. Tukano, Amazonas
C. a. albifrons/unicolor 241 Bra. Santa Sofia, Amazonas
C. a. albifrons/unicolor 265 Per. Genaro Herrera,Ucayali River
C. a. aequatorialis Ecu. Jama, Manabi Pacific
C. a. aequatorialis 17 Ecu. Manabi Pacific
C. a. yuracus 179 Ecu. Pastaza
C. a. yuracus 24 Ecu. Zamora
C. o. castaneus French Guiana, Cayenne
C. a. albifrons/unicolor 5 Bra. Branco River, Acre
C. a. albifrons/unicolor 6 Bra. Branco River, Acre
C. a. yuracus 90 Ecu. Pastaza
C. a. yuracus 115 Per. Tingo Maria, Huánuco
C. a. yuracus 198 Ecu. Pastaza
C. a. albifrons/unicolor 83 Col. Caquetá, Amazonas
C. a. albifrons/unicolor 81 Col. Caquetá, Amazonas
C. a. albifrons/unicolor 45 Col. Caquetá, Amazonas
C. a. yuracus 190 Ecu. Pastaza
C. a. albifrons/unicolor 249 Col. Santa Sofia, Amazonas
C. a. albifrons/unicolor 155 Col. Caquetá, Amazonas
C. a. yuracus 6 Ecu. Napo River
C. a. yuracus 33 Ecu. Napo River
C. a. albifrons/unicolor 207 Col. Amazonas
C. a. albifrons/unicolor 211 Col. Amazonas
C. a. albifrons/unicolor 62 Col. Amazonas
C. a. albifrons/unicolor 25 Col. Amazonas
C. a. albifrons/unicolor 27 Col. Amazonas
C. a. albifrons/unicolor 4CRO Col. Leticia, Amazonas
C. a. albifrons/unicolor 19 Col. Leticia, Amazonas
C. a. yuracus 186 Ecu. Pastaza
Undescribed Subspecies 22 Per. San Martín, Tarapoto
C. a. yuracus 208 Ecu. Pastaza
C. a. yuracus 13 Ecu. Napo
C. a. yuracus 17 Per. Pachitea River
C. a. yuracus 183 Per. Ucayali River
C. a. yuracus 301 Per. Pachitea River
C. a. albifrons/unicolor 110 Col. Leticia, Amazonas
C. a. yuracus 303 Per. Pachitea River
C. a. yuracus 199 Ecu. Pastaza
C. a. yuracus 302 Per. Pachitea River
C. a. yuracus 87 Ecu. Pastaza
C. a. yuracus Per. Ucayali River
C. a. yuracus 209 Ecu. Morona, Santiago
C. a. yuracus 236 Per. Sta. Clotilde, Napo
C. a. yuracus 86 Ecu. Napo
C. a. yuracus 193 Ecu. Pastaza
C. a. yuracus 9 Ecu. Napo
C. a. yuracus 5 Ecu. Misahualli, Napo
C. a. yuracus 7 Ecu. Pastaza
C. a. yuracus 201 Ecu. Pastaza
C. a. yuracus 210 Ecu. Pastaza
C. a. yuracus 185 Ecu. Morona, Santiago
C. a. yuracus 207 Ecu. Morona, Santiago
C. a. yuracus 88 Ecu. Napo
C. a. yuracus 82 Ecu. Napo
C. a. yuracus 088 Ecu. Pastaza
C. a. albifrons 2 Col. Palo Blanco, Arauca
C. a. yuracus 164 Ecu. Pastaza
C. a. albifrons/unicolor 169 Col. Inirida River, Guainia
C. a. albifrons 97 Col. Palo Blanco, Arauca
C. capucinus 75 Col. Magangué, Bolivar
C. capucinus 95 Col. Antioquia
C. capucinus 108 Col. Antioquia
C. capucinus 100 Col. Antioquia
C. capucinus 106 Col. Antioquia
C. capucinus 252 Col. Chocó
C. capucinus 251 Col. Chocó
C. capucinus 102 Col. Antioquia
C. capucinus 97 Col. Antioquia
C. capucinus 93 Col. Antioquia
C. capucinus 90 Col. Antioquia
C. capucinus 33 Col. Cauca
C. capucinus 89 Col. Antioquia
C. capucinus 98 Col. Antioquia
C. capucinus 109 Col. Córdoba
C. a. versicolor/C. capucinus (Hybrid) 186 Col. Sucre
C. capucinus 10 Col. Valle del Cauca
C. capucinus 26 Col. Chocó
C. capucinus 185 Col. San Marcos, Sucre
C. capucinus 186 Col. San Marcos, Sucre
C. a. albifrons 795 Col. Palo Blanco, Arauca
C. a. albifrons/unicolor 89 Bra. Indaduba, Amazon
C. capucinus 88 Col. Antioquia
C. capucinus 3 Col. Antioquia
C. capucinus 4 Col. Antioquia
C. capucinus 33 Col. Antioquia
C. capucinus 2 Col. Antioquia
C. capucinus 56 Col. Magdalena
C. capucinus 110 Col. Córdoba
C. capucinus 94 Col. Antioquia
C. capucinus 99 Col. Antioquia
C. capucinus 104 Col. Antioquia

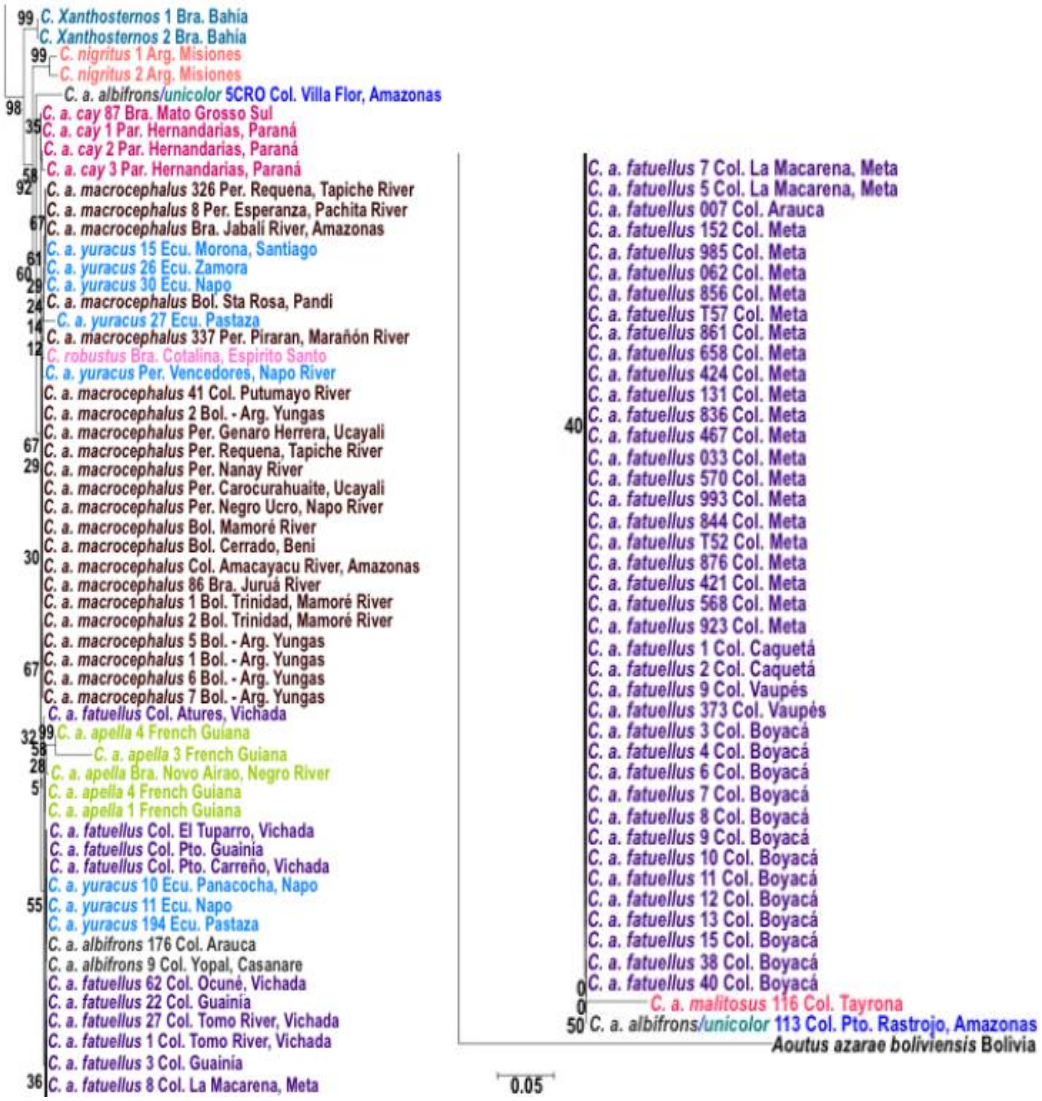


Figure 1. Neighbor-Joining tree with the Kimura (1980) 2P genetic distance with the 452 gracile and robust capuchin monkeys for four concatenated mitochondrial genes (control region, *COI, COII* and *Cyt-b*). The number in the nodes are bootstrap percentages. With *C. xanthosternos* and after begins the robust capuchin's clade. It's observable that within the robust capuchin's clade there are 13 individuals of different taxa of *C. albifrons* (*albifrons/unicolor*, *yuracus* and *malitosus*).

Molecular Procedures

The DNA from muscle and blood was extracted using the phenol-chloroform procedure (Sambrook et al., 1989), while DNA samples from hair and teeth were extracted with 10% Chelex resin (Walsh et al., 1991). The 452 capuchin individuals sampled were sequenced for four mt genes (*COI*, *COII*, *Cyt-b* and *D-loop*). For the mt *COI* amplification (polymerase chain reaction, PCR), we used the forward primer LCO1490 (5'-GGTCAACAAATCATAAAGATATTGG-3'), and the reverse primer HCO2198 (5'-

TAAACTTCAGGGTGACCAAAAAATCA-3') (657 base pairs, bp) (Folmer et al., 1994) under the following PCR profile: 94°C for 5 min, followed by 39 cycles of 94°C for 30 s, 44°C for 45 s, 72°C for 45 s, and a final cycle of 72°C for 5 min. For the amplification of the mt *COII* gene (located in the lysine and asparagine tRNAs) we used the forward primer L6955 (5' -AACCATTTCATAACTTTGTCAA-3') and the reverse primer H7766 (5' -CTCTTAATCTTTAACTTAAAAG-3') (720 bp) (Ashley and Vaughn, 1995; Collins and Dubach, 2000a; Ruiz-Garcia et al., 2010, 2012a,b). We used the following temperatures: 95°C for 5 min, 35 cycles of 45 s at 95°C, 30 s at 50°C and 30 s at 72°C and a final extension time for 5 min at 72°C. For both genes, the PCRs were performed in a 25 µl volume with reaction mixtures including 4 µl of 10 x buffer, 6 µl of 3 mM $MgCl_2$, 2 µl of 5 mM dNTPs, 2µl (8 mM) of each primer, 2 units of Taq DNA polymerase, 5 µl of ddH_2O and 2 µl (20–80 ng/µl) of DNA. The mt *Cyt-b* was amplified by PCR using the procedure of Montgelard et al., (1997) (1,140 bp) and the mt *D-loop* was amplified using the primers L15400 (5'-TCCACCATTAGCACCCAAAG-3') and H15940 (5'-CCTGAAGTCGGAACCAGATG-3') (610 bp) (Kocher et al., 1989). The conditions for *Cyt*-b and *D-loop* amplification were performed in 25 µl reactions including 2 µl of DNA, 2 µl of 10 x buffer, 13 µl ddH_20, 2 µl (25 mM) $MgCl_2$, 1 µl (10 mM) each of forward and reverse primers, 2µl (10 mM) dNTPs, and 2 units of Taq DNA polymerase. The standard thermal cycling program consisted of 10 min at 95°C, 35 cycles of 35 s at 94°C, 35 s at 55°C and 30 s at 70°C and a final extension time for 10 min at 72°C. The total length of the sequences studied were 3,127 bp.

All amplifications, including positive and negative controls, were checked in 2% agarose gels. The gels were visualized in a Hoefer UV Transilluminator. Both mt DNA strands were sequenced directly using BigDye Terminator v3.1 (Applied Biosystems, Inc.). We used a 377A (ABI) automated DNA sequencer.

For a subset of 27 individuals, DNA of high quality was extracted and isolated with the QIAamp DNA Mini Kit (Qiagen, Inc.) for blood samples (DNA Purification from blood or body Fluids; Spin Protocol) and for muscle samples (DNA Purification from tissues). To carry out the amplifications of the mt DNA of the capuchin specimens studied, we used the LongRange PCR Kit (Qiagen, Inc.), with final volume reactions of 25 µl. The reaction mix was composed of a 80-200 ng DNA template, 2 units of Long-Range PCR Enzyme, 3 µl of 10 x LongRange PCR Buffer, 4 µl (15 pmol) of each primer, and 2 µl of 10 mM dNTPs. The cycling conditions were as follows: 95°C for 3 min, followed by 50 cycles denaturing at 95°C for 20 s, primer annealing at 53–58°C (depending on primer set, with a decrease of 0.1°C every cycle) for 30 s, and extension at 72°C for 10 min. This was followed by 30 cycles of denaturing at 95°C for 20 s, annealing at 48–53°C (depending on primer set) for 30 s, and extension at 72°C for 5 min, with a final extension at 72°C for 10 min. Using four sets of primers to generate overlapping amplicons from 3,345 bp to 5,049 bp in length, allowed us to carry out a quality test for genome circularity (Bensasson et al., 2001; Thalman et al., 2004). There was an increased possibility of the reduction of amplifying nuclear mitochondrial pseudogenes (numts) in our analyses. Herein, we show the results of 16 mitochondrial genes (two rRNA, *D-loop* and 13 protein codifying genes; *D-loop*, 610 bp 12S *rRNA*, 930 bp; *16S rRNA*, 1,580 bp; *ND1*, 950 bp; *ND2*, 1,035 bp; *COI*, 657 bp; *COII*, 720 bp; *ATP8*, 205 bp; *ATP6*, 695 bp; *COIII*, 775 bp; *ND3*, 340 bp; *ND4L*, 305 bp; *ND4*, 1,380 bp; *ND5*, 1,810 bp; *ND6*, 530 bp and *Cyt-b*, 1,140 bp). The sequences were concatenated by means of the SequenceMatrix v. 1.7.6 (Vaidya et al., 2011). Thus, our analyses were undertaken with

13,662 bp which represents about 80-85% of the total mitochondrial DNA length. Overlapping regions were examined for irregularities, such as frameshift mutations and premature stop codons. The lack of such irregularities agrees quite well with the absence of numt sequences in our mitochondrial sequences. We used a 377A (ABI) automated DNA sequencer. The samples were sequenced in both directions to ensure sequence accuracy.

Data Analyses

We used the Kimura 2P genetic distance (Kimura, 1980) to determine the percentage of genetic differences between the alleged *Cebus* and *Sapajus* genera, among different species within different genera (*Alouatta*, *Ateles*, *Aotus* and *Saguinus*) and among different genera (*Alouatta*, *Lagothrix*, *Ateles*, *Aotus*, *Saimiri* and *Saguinus*). The Kimura 2P genetic distance is a standard measurement for barcoding tasks (Hebert et al., 2003, 2004). In the current work, we only show these genetic distances with the mt *COII* gene because the other genetic distances will be shown elsewhere. For Neotropical primates and Hominoidea, there is an average genetic distance at the *COII* gene of around 5.82% $\pm$ 1.64% among species within a genus, around 2-4% for subspecies within species and around 15.68% $\pm$ 1.73% among genera (Ascunce et al., 2003; Collins and Dubach, 2000b).

To analyze the genetic relationships among the 452 capuchin individuals for four mt genes as well as for 27 capuchin individuals for 16 mt genes studied, we used two neighbor-joining (NJ; Saitou and Nei, 1987) trees with the Kimura 2P genetic distance. Other phylogenetic trees are shown elsewhere, but the results were basically identical.

We used the Network 4.6.10 Software (Fluxus Technology Ltd.) to form a median joining network (MJN) to estimate possible divergence times among the haplotypes (joined *COI*, *COII*, *Cyt-b*, and *D-loop* sequences) in capuchin monkeys (Bandelt et al., 1999). The ρ statistic (Morral et al., 1994) was estimated and transformed into years. To determine the temporal splits, it is necessary to estimate the mutation rate at these mt genes. For mt *COI*, an average mutation rate of 1% per million years was employed (Matzen da Silva et al., 2011; Olson et al., 2009). This represents an average of one mutation each 152,000 years. Ruvolo et al., (1991) determined a mutation rate of 0.85% per million years per lineage for Hominoidea at mt *COII*. This represents one mutation on average each 199,402 years. This mutation rate was practically identical to that determined by Ruiz-García and Pinedo (2010) in a *Lagothrix* study (one mutation on average every 191,000 years). Similarly, for *Aotus*, Ashley and Vaughn (1995) and Ruiz-García et al. (2011) determined one mutation on average every 199,000 years at this same mitochondrial gene. Thus, we have used an average of one mutation each 195,000 years for *COII*. For mt *Cyt-b* in mammals, Nabholz et al., (2008, 2009) estimated a mutation rate of 2.5×10^{-2} substitutions/site/million years, which was equal to about one mutation each 120,482 years. We employed this mutation rate for this gene. For the mt *D-loop*, we employed a mutation rate of 7.05% per million years per linage following Forster et al., (1996), Geraldes et al., (2008), Hardouin et al., (2010), Heyer et al., (2001), Horai et al., (1995), Rajabi-Maham et al., (2008), Savolainen et al., (2002), Tamura and Nei (1993) and Ward et al., (1991), with different mammalian species. This represents one mutation on average each 40,000 years. Henceforth, we used an average of one mutation each 127,000 years for all four mt genes. In this analysis, we have employed only the haplotypes

represented by more than one individual, with the exception of those extremely important haplotypes, to more easily view the relationships among the different taxa of capuchins.

RESULTS

In Table 2 we compare the Kimura 2P genetic distance among taxa of *Cebus* and *Sapajus* but only for mt*COII*. The values between *C. albifrons* and *S. xanthosternos*, *S. nigritus* and *S. apella* were 6.0%, 7.9% and 6.7% (mean: 6.87%), respectively. *C. capucinus* showed the following values in regards to the same *Sapajus* species: 7.1%, 8.9% and 7.8% (mean: 7.93%), respectively. Clearly, all these values are closer to the average genetic distance for species (5.82% $\pm$ 1.64%) than to the average genetic distance for genera (15.68% $\pm$ 1.73%) for this gene. Within each one of these taxa, *C. albifrons* and *C. capucinus* (2.9% and 3.0%, respectively) showed considerably higher internal genetic diversity than *Sapajus* (*S. xanthosternos*, 0.1%; *S. nigritus*, 1.5% and *S. apella*, 0.4%, respectively).

Table 2. Kimura 2P genetic distance (percentage) and standard deviation among the different capuchin taxa studied by means of the mitochondrial *COII* gene. 1 = *Cebus albifrons*; 2 = *C. capucinus*; 3 = *C. nigritus*; 4 = *C. apella*; 5 = *C. xanthosternos*

Capuchin taxa	1	2	3	4	5
1	-	0.4	0.8	0.7	0.8
2	3.9	-	0.9	0.8	0.8
3	7.9	8.9		0.6	0.8
4	6.7	7.8	3.3	-	0.6
5	6.0	7.1	5.1	2.8	-

Table 3. Kimura 2P genetic distance (percentage) and standard deviations among the capuchin genera, *Cebus* and *Sapajus*, and other Neotropical primate genera (*Alouatta, Aotus, Ateles, Lagothrix, Saimiri* and *Saguinus*) studied by means of the mitochondrial *COII* gene. 1 = *Lagothrix*; 2 = *Alouatta*; 3 = *Aotus*; 4 = *Ateles*; 5 = *Cebus albifrons*; 6 = *Cebus* (= *Sapajus*) *apella*; 7 = *Saimiri*; 8 = *Saguinus*. In bold, the lowest genetic distance, which corresponds to *Cebus* and the alleged *Sapajus*

Genera	1	2	3	4	5	6	7	8
1	-	1.4	1.5	1.3	1.7	1.7	1.6	2.4
2	15.6	-	1.5	1.4	1.5	1.6	1.7	2.5
3	14.9	15.6	-	1.6	1.5	1.7	1.7	2.3
4	12.4	13.1	15.1	-	1.5	1.6	1.7	2.1
5	19.9	17.2	16.7	14.7	-	0.8	1.7	2.0
6	18.2	15.8	16.2	13.5	**5.8**	-	1.8	2.1
7	14.9	16.2	14.5	14.3	18.2	16.4	-	2.4
8	27.2	28.8	25.4	26.4	27.2	27.0	27.9	-

We analyzed genetic distances with our own sequences for other genera and species of Neotropical primates to compare with the previous results for *Cebus* and *Sapajus* (Table 3). The average genetic distance for different species of *Alouatta* was 7.24% $\pm$ 1.38%. For

different species of *Aotus* it was 4.7% ± 1.31%, for different species of *Ateles* it was 3.20% ± 0.77% and for different species of *Saguinus* it was 6.87% ± 3.01%. With the exception of the *Ateles* species, all the other values of species within genera were not significantly different from the average value between *Cebus* and *Sapajus* (5.8% ± 0.8%). Even the values within *Alouatta* and within *Saguinus* were higher than between *Cebus* and *Sapajus*. Similarly, the genetic distance between both alleged capuchin monkey genera is significantly lower than the average genetic distance among *Alouatta, Lagothrix, Ateles, Aotus, Saimiri* and *Saguinus* (18.64% ± 5.27%; this value is 3.2 times higher than the genetic differences between *Cebus* and *Sapajus*). Meanwhile the gracile capuchin group showed the highest genetic diversity of the all the taxa we studied (3.5%; Table 4), the robust capuchin group (0.4%) is even internally less differentiated than the genetic diversity found in single species of restricted distribution such as *Saguinus leucopus* (1.3%) or in *Saguinus oedipus* (1.1%). Therefore, the percentages of the genetic distances found did not justify *Sapajus* as a different genus from *Cebus*. Additionally, *Cebus* showed higher genetic diversity than that obtained in *Sapajus*, which indicates *Cebus* could be an original taxon and *Sapajus* deriving from it.

The neighbor-joining tree, with the Kimura 2P genetic distance (Figure 1) and four mt genes, showed some extremely interesting features. They do not agree with the previous view of Alfaro et al., (2012a, b), Bobli et al., (2012) and Nascimento et al., (2015). For example, there is not reciprocal monophylia between the two alleged *Cebus* and *Sapajus* genera. Meanwhile in the clade of *Cebus*, only sequences of *C. albifrons*, *C. capucinus* and *C. olivaceus* are present, the *Sapajus* clade contains sequences of *C. albifrons*. This result does not support the existence of two well separated genera.

Table 4. Mitochondrial nucleotide diversity (π, in percentage) and standard deviation (S.D) in capuchin monkey taxa and other Neotropical primate taxa

Genera	π	± S.D
Cebus albifrons	3.5	0.4
Cebus apella	0.4	0.1
Lagothrix	1.5	0.2
Ateles	0.4	0.1
Alouatta	1.5	0.2
Aotus	2.8	0.3
Saimiri	0.2	0.1
Saguinus leucopus	1.3	0.2
Saguinus oedipus	1.1	0.3

Within the *Cebus* clade, haplotypes of *C. albifrons, C. capucinus* and *C. olivaceus* were intermixed, not conforming clades correlated with the traditional morphological subspecies or species of gracile capuchins. The first branch to diverge within this clade were those corresponding to the Colombian III *C. capucinus* haplogroup (Ruiz-García et al., 2012; Ruiz-García and Castillo, 2016) together with some *C. albifrons* haplotypes from the area of Arauca (Eastern Llanos), Guainia (transition area from Eastern Llanos to Amazon) in Colombia (*C. a. albifrons*) and Negro River (Brazil) (*C. a. albifrons-unicolor*). The next haplotypes to diverge were *C. albifrons* from the Arauca region (*C. a. albifrons*) and from the most western Amazon areas where this species lives (Ecuadorian Amazon; *C. a. yuracus*).

The next cluster to diverge was comprised of *C. albifrons* individuals from the Western Amazon (Ecuador, Peru and Colombia; the most divergent haplotypes, *C. a. albifrons/unicolor*; *C. a. yuracus/cuscinus*, within this group were from the Ecuadorian Amazon). This cluster was in the origin of two small sub-clusters within it. One cluster had four animals from the Pacific Ecuadorian coast (traditionally named *C. a. aequatorialis*) and the other contained two *C. a. albifrons* from the Acre region (Brazilian Amazon) and the *C. olivaceus castaneus* individual from French Guiana. Later another large cluster appeared and it was integrated by individuals of *C. albifrons* from the Colombian departments of Santander, Norte de Santander, Boyaca and Arauca and they were identified following the traditional nomenclature as *C. a. adustus* and *C. a. leucocephalus*. Within this cluster there also appeared some exemplars of an Amazon origin. It also contained two individuals of *C. capucinus* from the vicinity of Buenaventura in the Pacific area of Colombia and that they were not included in the three Colombian haplogroups detected by Ruiz-García et al., (2012, 2016a).

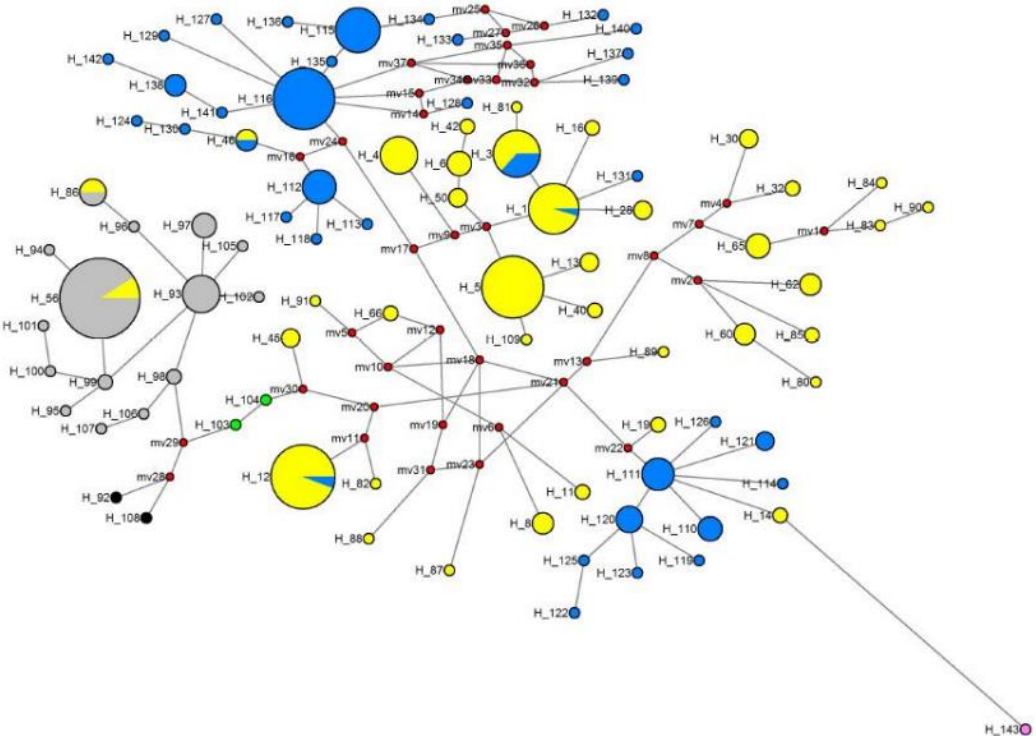


Figure 2. Median Joining Network (MJN) with haplotypes found at the four concatenated mitochondrial genes (control region, *COI*, *COII* and *Cyt-b*) gene for a fraction of the 452 capuchin monkeys analyzed. Yellow circles = all the individuals with *Cebus albifrons*'s morphotypes; blue circles = all the individuals with *Cebus capucinus*'s morphotypes; green circles = *Sapajus xanthosternos*; black circles = *Sapajus nigritus*; grey circles = all the individuals with *Sapajus apella*'s morphotypes (including *apella, cay, fatuellus, macrocephalus, pallidus, robustus*); pink circle = *Aotus azarae boliviensis* as an outgroup. Red circles indicate missing intermediate haplotypes. Clearly, the haplotypes of *C. albifrons* and *C. capucinus* were intermixed and the robust capuchin haplotypes are nested within the gracile capuchin haplotypes. Some *C. albifrons*'s haplotypes were mixed with some robust capuchin haplotypes. These results disagree with *Sapajus* as a differentiated genus from *Cebus*.

The next group to diverge was mainly composed of the Colombian II *C. capucinus* haplogroup together with two *C. albifrons* from the Amazon (Ecuador and Colombia; *C. a. yuracus* and *C. a. albifrons*). The next clade to appear was exclusively integrated by Central American *C. capucinus* individuals from Panama, Costa Rica and Guatemala. Ruiz-García et al., (2012) and Ruiz-García and Castillo (2016) affirmed that the Colombian II haplogroup seems to be more related to the Central American *C. capucinus* haplogroup. Later, a group composed by individuals with phenotypes of *C. a. versicolor* from the Colombian Santander Department appeared. Following this cluster, the next group consisted of mixed individuals from the Colombian Vaupes department (*C. a. albifrons*) and individuals from the Antioquia and Cordoba departments (*C. a versicolor*). Later, the main cluster containing animals with phenotypes of *C. a. versicolor* from the Colombian Tolima, Quindio, Risaralda, Santander, Antioquia, Bolivar and Boyaca Departments formed, including one individual from Vaupes (*C. a. albifrons*) and another from the Peruvian Amazon (*C. a. cuscinus*). The last and most recent cluster was formed by individuals of *C. a. versicolor* (Antioquia, Tolima, Quindio, and Boyaca Departments), *C. a. cesarae* (Magdalena and Cesar Department), *C. a. pleei* (Bolivar department), and one natural hybrid individual of *C. a. pleei* and *C. capucinus* from the San Jorge River. This last cluster also contained one individual of the Arauca Department, which was not phenotypically classified in either of the recognized morphological subspecies, one animal from the Colombian Amazon (*C. a. albifrons*) and the *C. capuchinus* individuals from the Colombian I haplogroup detected by Ruiz-García et al., (2012). Thus, this clade did not contain monophyletic groups which were correlated with the classical morphological species, *C. capucinus*, *C. albifrons* and *C. olivaceus*. As reproductive cohesiveness has been observed in the captivity and in the wild (hybrids) and similar chromosomal rearrangement are found among all these taxa as we will discuss in brief, we believe that this clade could be composed by a unique species or super-species in the geographical area we studied: *Cebus capucinus*. That is, we consider this clade as a unique species with multiple mitochondrial lineages, where multiple events of migration and mixture occurred and not a genus with multiple species as proposed by Bobli et al., (2012).

In the *Sapajus* clade, the first taxon to diverge was *S. xanthosternos*, followed by the divergence of *S. nigritus*. In both cases, these two clusters were highly significant (99% bootstraps). The following divergent cluster was integrated by an individual of *C. a. albifrons* from the Colombian Amazon. The next cluster to diverge was composed by the four individuals of *S. apella cay* (Paraguay and southern Brazil). Later, a big cluster appeared integrated by closely related animals we named *S. a. macrocephalus* (Southern Colombian Amazon, Western Brazilian Amazon, Peru, Bolivia and Northwestern Argentina), with one *S. a. robustus* (from Eastern Brazil). Interestingly, in this cluster, five *C. a. yuracus* (four from the Ecuadorian Amazon and one from the Napo River in Peru) were integrated. The last and more recent diverging clade was composed of two sub-clusters, both north of the Amazon River (all the other *Sapajus* taxa were from the Atlantic coast of Brazil and south of the Amazon River). *S. a. fatuellus* was from the western area from the Negro River (Colombia), and *S. a. apella* was from the eastern area of the Negro River (Brazil, Guianas). Also, in this *Sapajus* clade, seven *C. albifrons* were enclosed, three from the Napo River in the Ecuadorian Amazon (*C. a. yuracus*), one from the Colombian Amazon (*C. a. albifrons*), two from the Colombian Eastern Llanos (*C. a. albifrons*) and the unique individual of *C. a. malitosus* (Northern Colombia). Therefore, three well differentiated clades were determined within the *Sapajus* clade, *xanthosternos, nigritus* and *apella* (with different sub-clades in *apella*: *cay,*

macrocephalus, *fatuellus* and *apella*, with very small genetic distances among them). However, some *C. albifrons* haplotypes were intermixed with them. This agrees quite well with the fact that the gracile capuchins showed considerably higher levels of gene diversity than the robust capuchins and that, probably, the first was the origin of the second. In the discussion we offer explanations as to why *C. albifrons* haplotypes are intermixed with the haplotypes of the tufted capuchins. Note, this does not support *Sapajus* as a different genus from *Cebus*.

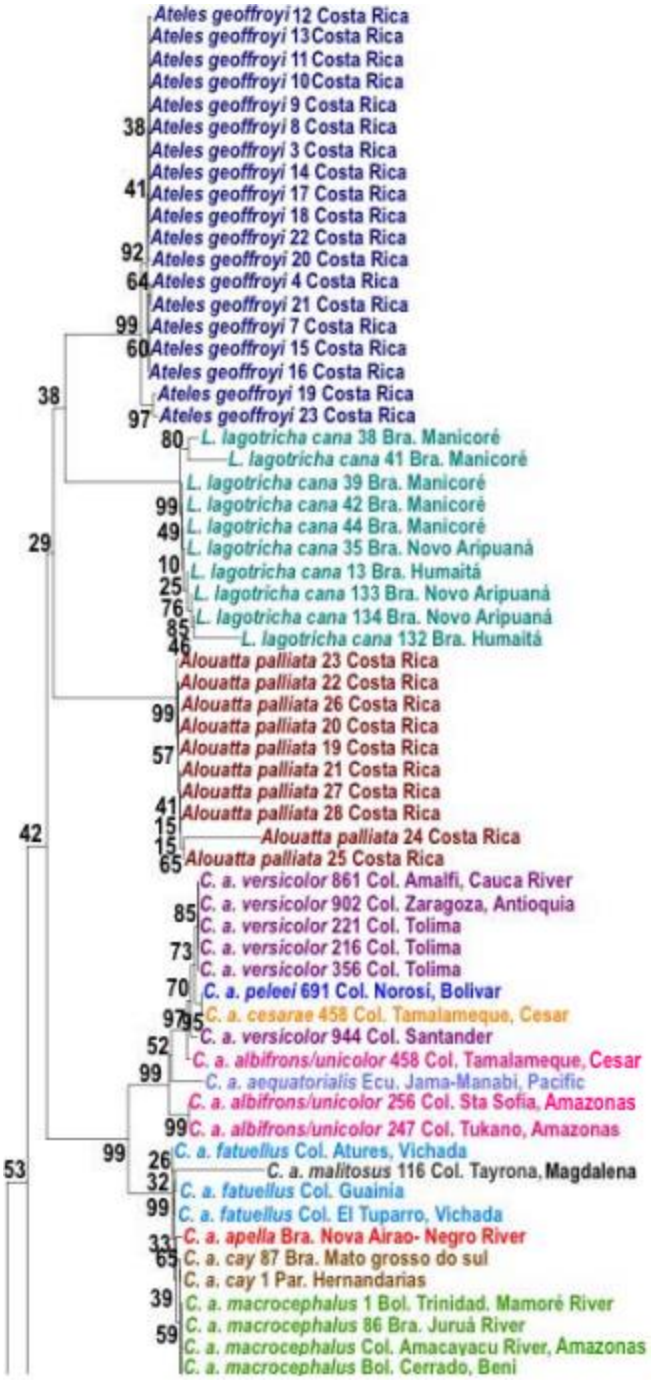


Figure 3. (Continued).

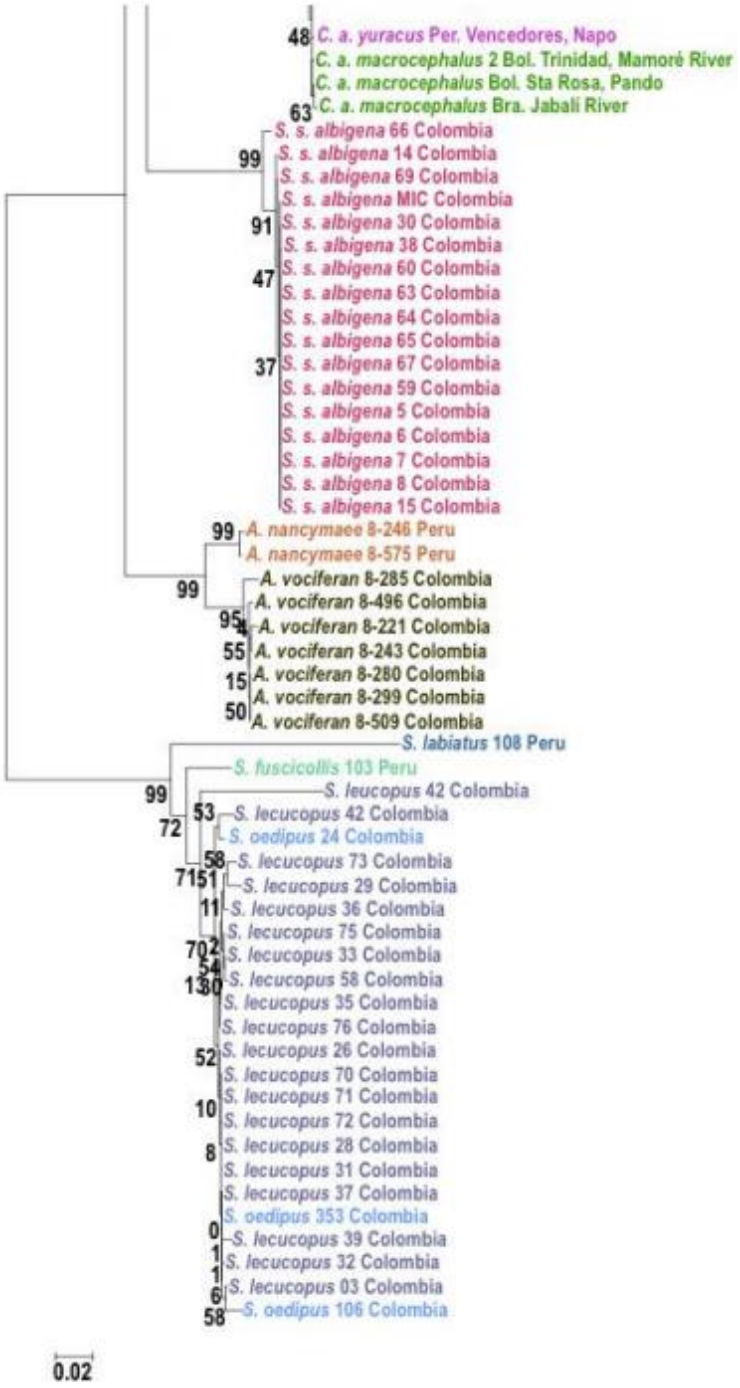


Figure 3. Neighbor-Joining tree with the Kimura (1980) 2P genetic distance with the 27 gracile and robust capuchin monkeys studied for 16 concatenated mitochondrial genes. Together the capuchin monkeys, other Neotropical primate species were sequenced for these 16 mitochondrial genes: *Ateles geoffroyi* (Costa Rica), *Lagothrix lagotricha cana* (Madeira River, Brazil), *Alouatta palliata* (Costa Rica), *Saimiri cassiquiarensis albigena* (Colombia), *Aotus nancymaee* (Peru), *Aotus vociferans* (Colombia), *Saguinus labiatus* (Peru), *Saguinus fuscicollis* (Peru), *Saguinus leucopus* (Colombia) and *Saguinus oedipus* (Colombia). The number in the nodes are bootstrap percentages.

The MJN procedure correlated very well with that described for the previous tree (Figure 2). The original and the most related capuchin haplotype with the outgroup haplotype (*Aotus*) corresponded to one haplotype found in two *C. albifrons* from the Arauca and Negro River areas (H14). This haplotype gave place to the Colombian III *C. capucinus* haplogroup. Relatively near to these haplotypes appeared a set of haplotypes (H65, H60 and related), which all belonged to the Western Amazon *C. albifrons*. In the central area of the MJN there appeared a set of haplotypes that belonged to *C. a. versicolor*, *C. a. cesarae-pleei*, *C. a. albifrons* (all from Colombia) together with *C. capucinus* of the Colombian I haplogroup. Indeed, H1 and H3 were shared by some of these *C. albifrons* and *C. capucinus*. In the upper area of the MJN, there are *C. capucinus* individuals from the Colombian II haplogroup, with H46 shared with two *C. albifrons* individuals form the Amazon (Ecuador and Colombia). Related with this haplotype set, there are the haplotypes of the Central American *C. capucinus*. In the lower part of the MJN, there are some dispersed *C. albifrons* haplotypes, all from the Western Amazon as well as the haplotypes of all the *C. a. adustus*-*C. a. leucocephalus* (H12) studied. This was shared with two *C. capucinus* from the Buenaventura area in Colombia. The most related haplotype (H45) of the gracile capuchins, which seems to give place to the robust capuchin haplotypes, was composed of three *C. albifrons* expanded in a wide area (Arauca and Guainia in Colombia and the Ecuadorian Amazon). The first robust capuchin haplotypes to appear were from *S. xanthosternos*, such as was revealed by the previous tree. From a yet undiscovered haplotype coming from *S. xanthosternos* the haplotypes of *S. nigritus* appeared. This seems to be an end branch without connections. There are also the haplotypes of *S. a. cay*, which in turn gave place to the most frequent *S. a. macrocephalus* haplotype (H93). One *S. a. macrocephalus* haplotype (H86) was shared with three *C. a. yuracus* from the Ecuadorian Amazon. The most recent robust capuchin haplotypes were those from *S. a. fatuellus* and *S. a. apella*. The most frequent *S. a. fatuellus* haplotype (H56) was shared with *C. albifrons* from the Colombian Eastern Llanos and from the Ecuadorian Amazon. Therefore, both analyses, tree and MJN, showed the exact same pattern of evolution in the capuchin monkeys. We estimated some noteworthy temporal splits among some relevant taxa of the capuchin monkeys. The oldest temporal splits within the capuchin monkeys always involved the Colombian III *C. capucinus* haplogroup and the *C. albifrons* haplotype (H14). For instance, the beginning of the splits between the Colombian III *C. capucinus* haplogroup with the Central American *C. capucinus* haplogroup or the split between the Colombian I *C. capucinus* haplogroup and the Central American one were 4.87 $\pm$ 0.57 MYA and 4.56 $\pm$ 0.55 MYA, respectively. The split of H14 and all the remaining Amazon *C. albifrons* haplotypes was around 4.92 $\pm$ 0.79 MYA. The temporal divergence between H14 and one of the main Western Amazon *C. albifrons*, from which many of the other *Cebus* lineages were generated, began around 3.76 $\pm$ 0.20 MYA. More recently, the temporal split from this Western Amazon *C. albifrons* with regard to some of the Northern Colombian *C. albifrons* haplogroups occurred around 3.05 $\pm$ 0.17 MYA (main *versicolor* clade), 2.48 $\pm$ 0.19 MYA (main *albifrons* clade from the Vaupes department), 2.73 $\pm$ 0.17 MYA (*adustus*-*leucocephalus* clade) and 3.33 $\pm$ 0.32 MYA (main *cesarae-pleei* clade). The temporal divergence from the original *C. albifrons*'s haplotype and the first diverging haplotype from the *Sapajus* clade was with one of the haplotypes of *S. xanthosternos* (around 3.31 $\pm$ 0.34 MYA). It was a similar temporal split that we commented about for the diverging process between the Western Amazon *C. albifrons* haplotypes and the different northern Colombian haplogroups of *C. albifrons* and *C. capucinus*. This result emphasizes that the

origin of *Sapajus* was throughout *Cebus* and it appeared more recently than the initial mitochondrial diversification of *Cebus capucinus-Cebus albifrons*. Indeed, a large fraction of the genetic diversity of *Sapajus* should be considered nested within *Cebus*, which does not agree with the view of both taxa as different genera. The temporal splits among some different relevant northern Colombian and Vaupes *C. albifrons* haplogroups were lower than the previous values and they were as follows: 1.13 ± 0.094 MYA (*adustus* and *leucocephalus* vs. *versicolor*), 1.13 ± 0.15 MYA (*adustus* vs. *cesarae-pleei*), 0.57 ± 0.14 MYA (*albifrons* Vaupes vs. *versicolor*), 0.88 ± 0.25 MYA (*albifrons* Vaupes vs. *cesarae-pleei*) and 0.39 ± 0.15 MYA (*versicolor* vs. *cesarae-pleei*). For the *Sapajus* clade, relevant temporal splits are those between *S. xanthosternos* and *S. a. cay* (2.08 ± 0.13 MYA) and *S. xanthosternos* and *S. a. macrocephalus* (3.24 ± 0.18 MYA). These values are considerably higher than those estimated among other *Sapajus* taxa as *S. a. cay-S. a. macrocephalus* (0.17 ± 0.17 MYA) or *S. a. fatuellus-S. a. apella* (0.19 ± 0.19 MYA). This agrees with the fact that *S. xanthosternos* should be differentiated as a species (and the same for *S. nigritus*) from the other *Sapajus* taxa. The molecular differentiation among the remaining *Sapajus* taxa is extremely recent (less than 0.2 MYA) which agrees with the fact that these taxa are, at most, subspecies of a unique species. Within some of these haplogroups, diversification began around 0.78 ± 0.10 MYA (Central American *C. capucinus*), 0.43 ± 0.14 MYA (Colombian II *C. capucinus* haplogroup), 0.23 ± 0.086 MYA (*versicolor*), 0.13 ± 0.084 MYA (*cesare-pleei*), 0.19 ± 0.12 MYA (*S. a. cay*), 0.15 ± 0.028 MYA (*S. a. apella*), 0.15 ± 0.08 MYA (*S. a. macrocephalus*) and only 3,500 YA in *S. a. fatuellus*. These temporal estimations clearly showed *S. apella* as being in the most recent diversification processes within the different groups.

Finally, we show a neighbor-joining tree with the Kimura 2P genetic distance (Figure 3) with 27 capuchin monkeys and some specimens of other genera (*Alouatta, Ateles, Lagothrix, Aotus, Saimiri* and *Saguinus*) for 16 mitochondrial genes. Although this tree is not as precise as that for the four mitochondrial genes because the number and variety of capuchin taxa is considerably lower, three main ideas are maintained. The first is that there is no reciprocal monophylia between *Cebus* and *Sapajus* because the last clade contains some individuals of *Cebus*, which disagrees with the fact that *Cebus* and *Sapajus* are two different genera. The second one is that the Amazon *C. albifrons* haplotypes originated the northern Colombian *C. albifrons*. Third, many of the morphologically defined *C. albifrons* taxa from northern Colombian are intermixed.

DISCUSSION

Why the *Cebus* Genus Should Not Substituted by the *Sapajus* Genus for the Robust Capuchins

Lynch-Alfaro et al., (2012b) proposed the division of capuchin monkeys into two genera, *Sapajus*, for the robust capuchins, and *Cebus*, for the gracile capuchins. They based this decision on alleged genetics, morphological, behavioral and ecological evidence. From a genetics point of view, the authors provided three main lines of evidence. 1- Capuchin monkeys were found to contain two well supported reciprocally monophyletic clades, the gracile capuchins (*Cebus*) and the robust capuchins (*Sapajus*); 2- The temporal divergence

between both alleged genera was estimated to have occurred during the late Miocene (around 6.5 MYA) and independent diversification within each of the two genera occurred during the Pliocene; and 3- There is an Amazonian origin for the gracile *Cebus* clade and an Atlantic Forest or Cerrado origin for the *Sapajus* clade.

However, our genetics results showed that these three lines of evidence for the split of the capuchin monkeys in two different genera are not so clear. In fact, they are incorrect when we include more individuals, localities, and mitochondrial genes sequenced. Here, we provide five findings which firmly contradict all three lines of evidence. 1- There is no reciprocal monophylia between the gracile and the robust capuchin clades. The main gracile capuchin clade did not contain any robust capuchin haplotype, but the robust capuchin clade is mixed with some gracile capuchin haplotypes; 2- The genetic diversity of the robust capuchins is generated from the gracile capuchins and therefore there is no independent diversification of both alleged genera; 3- The gracile capuchin evolution is much more complicated and old than that supposed by Lynch-Alfaro et al., (2012a, b); 4- The temporal split between both capuchin groups is lower than that estimated by Lynch-Alfaro et al., (2012a) ranging, based on our more complete data, from 5.5 to 3.3 MYA, depending on the procedures used. Indeed, as we will discuss later, the split of 3.3 MYA (MNJ procedure) is more probable than the split of 5.5 MYA (Bayesian procedure analyzed elsewhere). Thus, the divergence of both capuchin forms were during the Pliocene rather than in the Miocene as claimed by Lynch-Alfaro et al., (2012a,b) and 5- The genetic distances from relatively neutral molecular markers (which are the relevant characters to determine "real" phylogenetic relationships because are not affected by opportunistic adaptation or positive selection which can distort the "real" phylogenetic relationships between taxa) showed that the differences between gracile and robust capuchins groups are small. In other words the differences are less than those obtained for different species of Neotropical primates within the same genus. For instance, different species within *Alouatta* and *Saguinus* showed higher genetic distances than those of capuchin groups we analyzed. In addition, they were extremely and significantly lower than the values obtained between well differentiated related genera of Neotropical primates.

Therefore some of the fundamental genetics arguments put forth by Lynch-Alfaro et al., (2012a, b) for the separation of *Cebus* and *Sapajus* are not very consistent. It is also likely that many of the other arguments in favor of *Sapajus* by Lynch-Alfaro et al., (2012b) are weak and questionable. They employed, at least, five other argument lines for the split of *Cebus* and *Sapajus*:

A. They argued against Hershkovitz (1949) and Hill (1960), who clustered all robust capuchins as *C. apella*, and explained the greater genetic and species diversity in the robust group compared to the gracile group. From a genetic point of view, this is incorrect. For instance, only the animals classified "a priori" as *C. albifrons* showed 3.5% of nucleotide diversity, whist the animals classified as *S. apella* (including *cay, macrocephalus, apella* and *fatuellus*) only showed 0.4% of nucleotide diversity (near 9 times higher the nucleotide diversity in the gracile taxa than in the robust taxa). Another related point is that the coat and color characteristics could show more striking and visual differences in the robust clade than in the gracile one, but not in the molecular characteristics. We also highlight specific text of Lynch-Alfaro et al., (2012b) as weak proof for the splitting of capuchin monkeys into two different genera (…"An examination of capuchin monkey diversity, however, reveals far more

genetic and morphological difference *between* robust and gracile species than *within* either group"…). Perhaps this sentence could be expected between two different genera, but it could also be expected between two different species (with different subspecies) of the same genus. Thus, this sentence "per se" does not constitute any proof in favor of *Sapajus* as a different genus from *Cebus.*

B. There are many different morphological characteristics between gracile and robust capuchins. For instance, a list of these differences showed by Lynch-Alfaro et al., (2012b) are as follows. The robust capuchins are significantly more robust in terms of cranial and dental characteristics than the gracile capuchins (Ford and Corruccini, 1985). They have both cranial and postcranial specializations for the exploitation of hard and tough foods (Wright et al., 2009). Robust capuchins also have a more pronounced sexual dimorphism in cranial characters compared to gracile capuchins (Silva, 2001). Robust capuchin males have a sagittal crest that is lacking in gracile male capuchins (Silva, 2001). Byron (2009) compared *C. apella* with gracile capuchins and found that *C. apella* had a significantly more robust mandibular and temporal fossa morphology. Jungers and Fleagle (1980) showed body proportion differences in early development, in *C. albifrons* and in *S. apella* with divergent growth trajectories of limb length as a function of body mass. *C. albifrons* is longer-limbed in proportion to body mass than *S. apella* throughout development with this difference increasing as growth progresses.

The first problem with these morphological characters is that they could exist between species and they are not necessarily only existing between genera. One magnificent counterexample is that of two wild cats, the puma and the jaguarundi. Traditionally, they were considered two species belonging to two distant genera (*Puma concolor* and *Herpailurus yagouaroundi*) because, for instance, their sizes and weights are extremely different (head and body length is 505-645 mm and weight 4-9 kg for the jaguarundi and head and body length is 860-1,540 mm and weight 30-120 kg for the puma). However, molecular analyses (Johnson et al., 2006) showed that, despite the vast differences in size, the ancestors of both diverged around 4-5 MYA and now there is agreement among all the cat specialist to consider these species as part of the same genus (*Puma concolor* and *Puma yagouaroundi*). This example (which is absolutely opposite to the philosophy of Lynch-Alfaro et al., 2012b) raises a second question. The major part of these morphological characters do not provide any value from a phylogenetic perspective because they respond to quick adaptative and positive natural selection. There are many examples of this. Masterson (2001) reported that *S. nigritus* and *S. robustus* were both significantly larger than, and had significantly larger dental arcades, increased prognathism, and larger in absolute cranium size compared to *S. libidinosus*. This difference is so important especially in regards to *S. cay*, with adult crania that are so small, they look like the juvenile ones in *S. nigritus* and *S. robustus*. This shows that adaptation via natural selection occurred quickly and also operated within the robust capuchin group. Cáceres et al., (2014) performed an interesting series of geometric morphometric analyses of skull shape (23 homologous landmarks were digitized to describe skull shape) in 228 capuchin monkey individuals belonging to seven taxa of robust capuchins and two taxa of gracile capuchins representing 94 localities in South America. They regressed skull shape against latitude, longitude, skull size and

environmental variables. The main results of this work clearly showed that many craniometrics characteristics could be not used as phylogenetic characters because they are affected by convergent natural selection by similar environmental pressures. This suggests that there is no clear separation between the two alleged capuchin genera, and overlap occurred in the central area of the RW1/2 plot between species of both *Cebus* (*C. olivaceus* and *C. albifrons*) and *Sapajus*. The shape variance associated with RW2 described variation in the skull width and zygomatic arch position. Along this axis *S. nigritus* occupied extreme negative values whereas *S. apella* had extreme positive values. This shows that there were more differences between two taxa of *Sapajus* than between these *Sapajus* taxa with *Cebus* taxa. A second result showed Hotelling's pairwise comparisons indicated that *C. olivaceus* and *S. apella* were more related to each other and different from many other capuchin taxa in terms of skull shape. A third result showed that some tests resulted in significant differences between *S. macrocephalus* and *S. xanthosternos* as compared to *S. cay* and *S. libidinosus*. *S. macrocephalus* and *S. xanthosternos* were the species with the largest skulls, although they are not directly related as we show with molecular phylogenetic analyses. This is further proof that there is no validity of using size as a phylogenetic tool to separate *Cebus* and *Sapajus* into two different genera. A fourth result is related to skull allometry. No slope differences were recorded between genera (P = 0.230) and between species (P = 0.083). Thus, skull allometry has no significant phylogenetic signal. A fifth result showed that latitude has a significant impact on skull shape. Longitude always explained a smaller percentage of shape variance than latitude and its impact was non-significant. Specimens at southern latitudes were generally characterized by more elongated rostrum, and long and narrow skulls and tooth rows. The relative distance between the zygomatic arch and the rostrum tip was also proportionally small. Specimens collected near the equator had relatively shorter muzzles and wider skulls Species distributed in localities with low precipitation, low minimum temperature, and high seasonality exhibited skulls with more elongated muzzles, narrow skulls at the zygomatic arch and relatively larger teeth (*S. nigritus* and *S. cay*). Conversely, both *C. albifrons*, *C. olivaceus*, *S. apella* and *S. macrocephalus* showed positive scores in vector SW1 shape and had more robust skulls, smaller teeth, enlarged neurocrania and wide zygoma. This suggested that both alleged genera vary in skull shape along the same environmental gradient in an identical mode. As explained by Cáceres et al., (2014), during their Pleistocene radiation, new species within the robust capuchin clade moved to the north, and started to resemble (in skull shape) to the *Cebus* capuchins when they occupied the Amazon rain forest. The pool of Amazonian species includes two gracile capuchins and two robust capuchins. Although differences in skull shape between these species are significant, they all share a wide skull and a narrow muzzle. Interestingly, we find differences not only in skull shape but also in skull size between *S. macrocephalus* and *S. apella*, suggesting that these two taxa are morphologically distinct, in spite of the very small genetic distances between them (Ruiz-García et al., 2012b and this work). The same occurred with the two gracile capuchin taxa. It's apparent in the relative warp and PLS plots. *C. albifrons* is characterized by a relatively more elongated rostrum than *C. olivaceus*. Also the genetic distances between *C. albifrons* and *C. olivaceus* are very limited

(even *C. olivaceus* is nested within *C. albifrons* as it was showed in Ruiz-García et al., 2010 and here). In summary, partial least squares between bioclimatic variables and skull shape explained some 98% of the covariation between environment and shape. Species distributed in drier, more seasonal southern localities exhibit a narrow skull with elongated muzzle and relatively larger teeth. Variation partitioning suggests that the difference in skull shape between species is highly correlated with climatic variation but not with skull size. As robust capuchin taxa moved to the north, they adapted to the local environmental conditions, eventually resembling gracile capuchins in skull shape as they reached the Amazon rain forest, in response to their shared environmental conditions. All of these examples indicate that morphometric data are not helpful in determining phylogenetic relationships and systematic differences within capuchin monkeys.

Lynch-Alfaro et al., (2012b) claimed that in terms of cranial morphology, there are no characters that allow a researcher to have confidence in determining among *Pithecia*, *Chiropotes*, or *Cacajao* without also having known geographic origins of all samples. Conversely, several cranial characteristics lead to the easy differentiation between the gracile and robust capuchins even for a skull of unknown provenance that lacks any associated pelage data. This argument is misleading and does not bring forth evidence in favor of two different genera of capuchins monkeys. Obviously, natural selection pressures have affected the robust capuchins in their rapid expansion during the late Pleistocene. These varied ecological conditions during colonization are very different to the more homogeneous ecological conditions (and therefore more homogeneous natural selection pressures) in which evolved some genera as those they cited. For instance, we can perfectly discriminate an Asian human skull from human skulls in other areas of the world (Dirkmaat, 2012; Turner, 1976; Wade, 2014), but this doesn't mean that the Asian humans are from another genus than the rest of the humanity. In another example, no one confounds a skull of a Neanderthal from that of a current human (*Homo neanderthalensis* and *H. sapiens*, or, *H. s. neanderthalensis* and *H. s. sapiens*; Bilsborough, 1972; Finlayson et al., 2006; Finlayson and Carrión, 2007; Howells, 1974; Jelinek, 1976) because the qualitative and quantitative differences are striking. Yet, no one classifies these human forms in different genera. What is really important is the information we can obtain from neutral or quasi neutral areas of the genome and not from the flexible characters easily affected by adaptation natural selection. Furthermore, we ignore the genetics basis of many of these morphological differences and how much is the phenotypical plasticity of these characters.

C. Lynch-Alfaro et al., (2012b) also presented a list of some ecological and some behavioral differences between gracile and robust capuchins to justify them as different genera. Gracile capuchins have larger group sizes and larger home ranges and they are found at lower densities than the robust taxa when the two types are found in sympatry (Terborgh, 1983). Haugaasen and Peres (2005, 2009) found *S. macrocephalus* as a true habitat generalist, with high population density in all kinds of forest, whilst *C. albifrons* was found at a lower density than *S. macrocephalus* in terra firme forest, and rarely observed in the flooded forests, except at the height of the fruiting season. The difference in population density in the two species was

considered a result of the heavy reliance of *C. albifrons* on widely dispersed fruits such as figs. *S. apella* has a more generalized use of forest and edge habitats whilst *C. olivaceus* prefers high terra firme rainforest, avoiding forest edge in Surinam (Mittermeier and Van Roosmalen, 1981). Territoriality and range behavior also appear different between the two capuchin groups in areas of sympatry. In Manu National Park (Peru), *S. macrocephalus* groups primarily used a core area, with a relatively small home range, large overlap between groups, and peaceful interactions across groups. In contrast, *C. albifrons* groups aggressively defended a much larger, nearly exclusive territory and traveled longer distances, spending days in a particular part of the home range until the food sources were exhausted, and then moving on (Terborgh, 1983). Other difference between *Cebus* and *Sapajus* is in stone tool use in the wild. It would appear to be ubiquitous across robust capuchin populations in dry habitats (*S. flavius, S. libidinosus, S. xanthosternos*; Emidio and Ferreira, 2012), but it has not been recorded in other long term studies of *Sapajus* in rainforest conditions (*S. nigritus, S. apella* and *S. macrocephalus*). This can be explained in part because the alluvial floodplain of the central Amazon has no stones available for tool use (Spironello, 1991). However, these differences in ecological traits and behaviors related to ecological characteristics are not related to different genera. These differences could occur between species. Another clear counter-example is again related to wild cats. The ecology and behavior of lions (*Panthera leo*; for instance a social species) are extremely different to that of the leopards (*Panthera pardus*; for instance a solitary species) and yet there are no research claims that they form different genera (Ewer, 1973).

D. Another list of alleged differences to split *Cebus* and *Sapajus* by Lynch-Alfaro et al., (2012b) was that related with courtship, mating, and postcopulatory display. They claimed that *Sapajus* displays the richest repertoire of sexual and courtship behaviors ever described for nonhuman primates (Fragaszy et al., 2004). In contrast, *C. capucinus* females in Costa Rica exhibits little proceptive behavior but instead tended to look and behave the same during fertile and nonfertile periods (Carnegie et al., 2005). Identically, *C. albifrons* females in Ecuador never showed the extended proceptivity displays commonly observed in the robust capuchin taxa (Matthews, 2012). Additionally, in courtship, *Sapajus* males and females raise their brows up and back and "grin," pulling their lips back to expose their teeth in a grimace, whilst the gracile capuchins instead protrude their lips in a "duck face," which continues throughout copulation as well (Matthews, 2012; Perry, 2008). This extreme difference in reproductive signaling suggests reproductive isolation following Lynch-Alfaro et al., (2012b). However, as in the previous cases, the existence of reproductive isolation is basically one of the properties of what is a species more than what is a genus. They employed typical arguments to differentiate species by using BSC to differentiate genera. Furthermore, the existence of a wide range of sexual and courtship behaviors in the robust capuchins, as well as the use of tools, does not mean they are different genera from *Cebus*. There are even very different sexual and courtship behaviors in our own species (also submitted to very different environmental conditions in our natural history as in the robust capuchins), yet we don't claim the existence of different species or genera for humans. Also, no one

claims that variability in human sexual behavior is the reason we classify ourselves as a different genus from other Primates.

E. Curiously, Lynch-Alfaro et al., (2012b) practically mention nothing about the karyotype evolution of the capuchin monkeys. They literally cited the following: "….. The karyotypes of *Cebus* and *Sapajus* differ in fundamental number and diploid number, but the data at present do not allow for a coherent evolutionary analysis because of the blanket use of "*C. apella*" to describe the samples from the robust group…" As we will show later, there is a notable number of karyological results of the capuchin monkeys, which together the molecular results, allow us to reconstruct the evolutionary history of this group of monkeys. In fact, Nieves et al., (2011) demonstrated that karyological results agree with the capuchin monkeys as a single unit. They employed a heterochromatin probe for chromosome 11 of *C. libidinosus* (11qHe+ CLI probe), obtained by chromosome microdissection. When FISH experiments were analyzed, in all the capuchin monkeys that they employed, they found six to 22 positive signals among them (both gracile and robust capuchins) located in interstitial and telomeric positions. No hybridization signals were observed when the 11qHe+ probe was applied to over 14 other specimens of Ceboidea (*Alouatta*, *Aotus*, *Ateles*, *Saimiri*, *Callithrix*, *Callicebus*, *Leontopithecus* and *Pithecia*). Therefore, this FISH study confirmed the existence of a genus-specific extracentromeric heterochromatin in the capuchin monkeys (both gracile and robust), suggesting that this heterochromatin is the same but with a different amplification pattern among the different taxa of capuchin monkeys. This means that basically the karyological studies agree more with a unique genus of capuchin monkeys than with two genera.

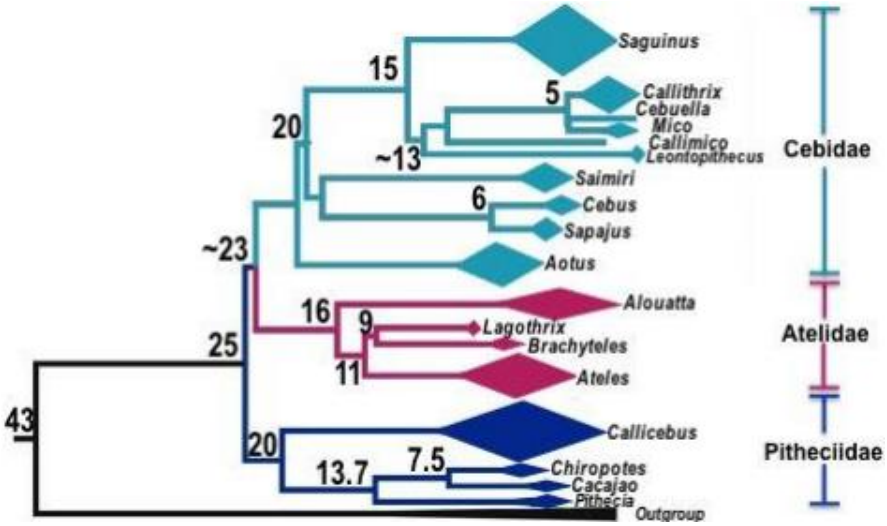


Figure 4 Dendrogram of the phylogenetic relationships of the current Neotropical primate genera extracted from Schneider and Sampaio (2015) with modifications. The figure shows that if *Cebus* and *Sapajus* are split, then other genera as *Saguinus*, *Aotus*, *Alouatta*, *Ateles* and *Callicebus* should also be split.

Another interesting question, with practical consequences, is that if we assume the existence of two genera of capuchin monkeys, then, other well resolved Neotropical primate genera must be divided into new genera as well. If we look at a modification of the phylogenetic tree of Schneider and Sampaio (2015) (Figure 4), it's clear that if *Cebus* and *Sapajus* are split, consequently, other genera as *Saguinus, Aotus, Alouatta, Ateles, Callicebus* and even *Callithrix* must also be divided into more (new) genera. Additionally, it seems logical that an indiscriminate application of PSC could generate an unmanageably large number of species and even of genera. We would be negligent to forget the great number of problems and confusion caused by using primitive typological classifications in the past (the case of the 86 supposed species of brown bears in North America alone following Merriam, 1918, to only cite one example). The primatologists who follow the PSC, upgrade all the primate subspecies to species, or, even, species to genera (as is the present case) but we consider that this is only moving the problem up one level as it obscures the reality of a real evolutionary unit. The BSC should not be ignored just because for some taxa it is not easily translated into an operational definition. However, in the current case, there are many results on molecular and karyological traits and reproductive natural events to apply the BSC for the capuchin monkeys. Indeed, many researchers, which currently employ molecular markers, however, are still using a typological view of the evolution because they did not understand the basic fundamentals of the Neodarwinism synthesis (Ayala, 1975, 1994a,b, 1995; Dobzhansky, 1937, 1970, 1973; Ford, 1976; Huxley, 1942; Mayr, 1942, 1963, 1977, 1978, 1992; Rensch, 1947, 1950; Simpson, 1944, 1953; Stebbins, 1950; Wright, 1968, 1969, 1977, 1978). For all of these reasons, and from this moment on, we just use the term *Cebus* for all taxa of capuchin monkeys in this work.

The Evolution of the *Cebus* Genus

The Evolution of the Gracile Capuchins: They Began the History of the Current Capuchins

Our analyses showed two possible beginnings for the initial evolution of the capuchin monkeys: 1- The original evolution of the capuchin monkeys began in the area of the Orinoco and Negro River (Orinoco and Northern Amazon; *C. albifrons*) which quickly generated the first wave of *C. capucinus* (Colombian III haplogroup) towards what is currently northwestern Colombia, or 2- The oldest capuchin monkey haplotypes were those of the Colombian III *C. capucinus* haplogroup, which in turn originated the first *C. albifrons* haplotypes in the Orinoco and Negro River. This molecular result is extremely noteworthy and agrees quite well with the karyological results of the genus *Cebus*, a fact which was not taken into account by Lynch-Alfaro et al., (2012a, b). Dutrillaux et al., (1986) showed that the ancestral karyotype for *Cebus* was *C. capucinus* with 54 chromosomes with 8-9 autosomic pairs being meta or sub-metacentric and with polymorphism in chromosome 19. The *C. albifrons*'s karyotype is very similar to that *C. capucinus*, which again agrees quite well with our molecular results to not differentiate *C. capucinus* and *C. albifrons* as two clear and well differentiated taxa. A few individuals of both taxa showed 52 chromosomes (Egozcue and Egozcue, 1966; Koiffmann and Saldanha, 1974). *C. olivaceus* shows 52 chromosomes (with the fusion of chromosomes 10 and 27 of *C. capucinus*). *C. apella* also shows 54 chromosomes (as in *C. capucinus* and many *C. albifrons*) with 10 autosome pairs as meta or

sub-metacentric. Three chromosome pairs are different in *C. apella* relative to *C. capucinus* (chromosomes 6, 13 and 23). Torres de Caballero et al., (1976) and Amaral et al., (2008) analyzed the interspecies chromosomal comparisons among the capuchin monkey, by using G- and Q-banding patterns. They suggested that *C. capucinus*, *C. albifrons* and *C. apella* share 19 chromosome pairs, *C. capucinus* and *C. albifrons* share 25 pairs and *C. capucinus* and *C. apella* share 20 pairs.

C. capucinus most resembles the putative ancestor, as all chromosomes found in *C. capucinus* are observed in *C. albifrons* and *C. apella*. Both, *C. albifrons* and *C. apella*, seem to have been derived from an ancestor with a karyotype similar to *C. capucinus*. The *C. capucinus* karyotype is clearly closer to *C. albifrons* than to *C. apella*. Figure 5, from Amaral et al., (2008), clearly shows this perception. *C. capucinus* occupied a more basal position, with a chromosomal composition very similar to the putative ancestral Platyrrhini karyotype, following Richard et al., (1996). The phylogenetic relationships of *C. capucinus* and *C. albifrons*, in relation to the ancestral karyotype, are not clearly defined. However, it is clear that the karyotype of some *C. albifrons* differ from that of some *C. capucinus* by a pericentric inversion in the 14/15 association, which results in a metacentric association 15/14/15/14. Amaral et al., (2008) identified a tandem fusion, followed by a pericentric inversion involving the homologous human chromosomes HSA15b and HSA8b, in the *C. albifrons* they studied. Sex chromosomes were similar in all the species of gracile and robust capuchin monkeys, with submetacentric X chromosome and a small acrocentric Y chromosome (Amaral et al., 2008). Within other genera of Neotropical primates, the karyotype diversity is considerably higher than in *Cebus* (for instance, *Aotus*, *Callicebus*, *Alouatta*, Koiffmann, 1982). Therefore, as it was summarized by De Oliveira et al., (2012), the oldest, and the putative ancestral Neotropical Primate karyotype (2n = 54) is from *C. capucinus*. The *C. apella* karyotype can be derived from this by an inversion 14/15/14 (García et al., 2000). It's not very differentiated from the ancestral karyotype, and therefore provides additional evidence against *Sapajus* as a differentiated genus from *Cebus*. Each species-specific fusion explains the reduction in diploid number to 52 in some *C. albifrons* (12/15) and *C. olivaceus* (8/15/8), respectively (Amaral et al., 2008).

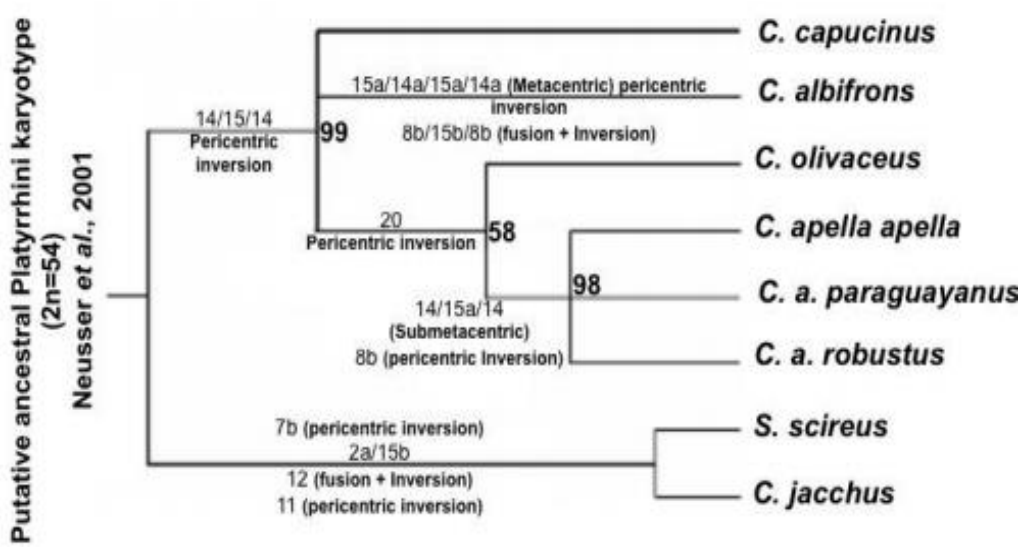


Figure 5. Chromosomal cladogram showing the relationships among different gracile and robust capuchin species. The tree is from the studies of Amaral et al., (2008) and Neusser et al., (2001).

The temporal divergence of these first *C. capucinus*-*C. albifrons* haplotypes occurred during the beginning of the Pliocene (5-4 MYA). Several important facts occurred in this epoch. For example, the last uplift of the Northern Colombian Andes happened 3-5 MYA reaching its current elevation of 4,000-6,000 meters above sea level (Andriessen et al., 1994; Gregory-Wodzicki, 2000; Van der Hammen, 1995; Van der Hammen et al., 1973). Such an event could be important for the initial fragmentation of the initial *C. capucinus*-*C. albifrons* haplotypes. Other larger Amazon changes could have also influenced the initial fragmentation of the original capuchins. Espurt et al., (2007, 2010) demonstrated that the Nazca Ridge subduction imprint had a significant influence on the eastern side of the Andes by means of the Fitzcarrald Arch. This uplift is responsible for the atypical three-dimensional shape of the Amazonian foreland basin. Related to the Nazca Ridge subduction, arc volcanism in the Peruvian Andes ceased around 4 MYA (Rosenbaum et al., 2005). Thus the Fitzcarrald Arch uplift is not older than 4 MYA. Indeed, this arch defines three drainage basins: northern Amazon rivers, eastern Amazon rivers, and southern Amazonian rivers (Madre de Dios River basin and Mamore-Beni River basin). Also at the beginning of the Pliocene (5 MYA), the sea level rose 100 m for a duration of 0.8 MY (Haq et al., 1987). Thus, marine transgressions could have also had a great influence on diversification of these initial capuchin taxa. Later, and independent of either origin hypothesis, a wide array of *C. albifrons* haplotypes was generated in the Western Amazon which, in turn, generated all the remaining groups of capuchin monkeys. The first major split in the gracile capuchins was detected by Bobli et al., (2012) and they dated this event to around 2.5 MYA (group A in their Figure 2) through the analysis of five specimens (one from Peru, two from Ecuador and two from Brazil). Clearly, the affirmation of Nascimento et al., (2015) about the necessity to sample a larger number of specimens, localities and even other genes to be confident with the origins and evolution of the genus *Cebus*, is verified here.

An artifact of the sampling design of Lynch-Alfaro et al., (2012a), is not being able to detect the oldest diversification process. Therefore, they concluded that the gracile capuchins appear to have radiated rapidly from an *albifrons*-like ancestor in the Amazon. Additionally, they concluded that dispersal of *C. capucinus* into Central America (≈1.9 MYA) occurred later than that of *Alouatta* and *Ateles* (Collins and Dubach, 2000a,b; Cortés-Ortiz et al., 2003). Based on this chronology, the capuchins were in Central America long after the completion of the Isthmus of Panama (2.8-3.5 MYA) (Nores, 2004; Coates and Obando, 1996). Apparently, this corresponds well with predictions by Ford (2006), who suggested that *C. capucinus* arrived in Central America in a 'second wave' of primate introductions around 2 MYA. However, our data, with many more exemplars from many additional geographical points, do not agree with this hypothesis. The splits between the Colombian *C. capucinus* haplogroups and the Central American capuchins oscillated from 4.87 to 3.7 MYA. If we take the temporal split between the Colombian II *C. capucinus* haplogroup and the Central American one as the most probable (3.7 MYA), this coincides with the formation of the Panama land bridge. Or, this coincides with the formation of the Chocó-Panamá island bridge slightly earlier (Galvis, 1980). This bridge could have been used by the ancestors of the current *C. capucinus* to colonize Central America from Northern South America. During the upper Pliocene orogeny, the present Tuira, Atrato, and Sinú basins as well as nearby lowlands were raised above sea

level. Thus, the mountains of Southern Central America and the northern Andes were uplifted to about their present elevation (Van der Hammen, 1961). Even if the divergence splits were around 4.5–5 MYA, the Nicaraguan, Panamanian and Colombian portals remained open (late Miocene-Middle Pliocene). Numerous volcanic islands existed from the lower Atrato Valley and the Tuira Basin of Eastern Panama to the Nicaraguan portal. These could have been used by the *C. capucinus* to migrate northward. The Cuchillo Bridge of the Urabá region, connecting the Tertiary Western Colombian Andes with the Panamanian islands was probably above sea level during this period. Simpson (1950, 1965) claimed that many mammals were ‘‘island hoppers,” and capuchin monkeys are extremely good colonizers. However, for central and Northern Panama, Ruiz-García and Castillo (2016) showed animals from the Colombian II *C. capucinus* haplogroup and the Central American *C. capucinus* haplogroup mixed in the same troops with no morphological differences among them. This demonstrates reproductive cohesiveness, which is proof against the claims of Bobli et al., (2012) that the Central American capuchin is another differentiated species of gracile capuchin, *C. imitator*.

We detected at least, five or even six large migration events, and probably many smaller migration events, (Bobli et al., 2012 only detected two of these migration events) related with the Western Amazon *C. albifrons* array, around 3.7-2.5 MYA (late Pliocene-beginning of Pleistocene):

A. One migration towards northern South America generated two or three different groups: 1- One generated a large fraction of the *C. albifrons* taxa in some areas of the Colombian Amazon and Central and Northern Colombia: *C. a. albifrons* from Vaupes, *C. a. versicolor, C. a. cesarae, C. a. pleei,* and the *C. capucinus* of the Colombian I haplogroup; 2- A second one generated the *C. capucinus* individuals from the Colombian II haplogroup and 3- directly from a *C. albifrons*’s wave or from the Colombian II *C. capucinus* haplogroup appeared the Central American *C. capucinus* haplogroup as we above explained.
B. Another migration towards Northern South America generated *C. a. adustus-C. a. leucocephalus* and the very limited geographically speaking *C. capucinus* lineage that we detected in Buenaventura.
C. Another migration from the Western Amazon array generated the Pacific Ecuadorian *C. albifrons* population (*C. a. aequatorialis*). Our MJN estimation showed that the *C. a. aequatorialis* haplotype (H7) diverged from an important Western Ecuadorian Amazon *C. albifrons* haplotype (H65) around 780,000 $\pm$ 112,600 YA. This Pacific *C. albifrons* in Ecuador is intriguing because of the elevation of the Central and Northern Andes 2 MYA. For instance, the Andean chain between Cajamarca and Huancavelica in Peru appeared by volcanism around 2 MYA and the temporal split between Amazon Ecuadorian *C. albifrons* haplotypes and those from the Pacific Ecuadorian areas is more recent. Certainly, some authors, for instance Hernández-Camacho and Cooper (1976) and Ruiz-García et al., (2006), mentioned the existence of a zoological passage east of the eastern Andes cordillera into the upper Magdalena valley in Colombia. Several species of Primates such as *Cebus apella, Lagothrix lagotricha lugens, Ateles belzebuth* and *Saimiri sciureus albigena,* as well as other vertebrate species, have used this passage. Maybe a similar passage existed in the Ecuadorian Andes at some point in the past. However, there is another possibility. The current Pacific Ecuadorian *C. albifrons* population could be related to *C.*

capucinus through the Pacific Coast from the north. Another alternative hypothesis could focus on the fact that this population was created by human action in more recent times.

D. Another migration route existed from the current Brazilian Amazon Acre going northeast towards Northern South America, generating *C. olivaceus*. This migration route was also detected by Bobli et al., (2012). They named a group (B) consisting of two parts (B.1 and B.2). B.1 contained two individuals, one from Barcelos on the right bank of the Negro River and the other from the Curanja River, upper Purus River in the Department of Loreto in Peru near the Brazilian border. B.2 contained individuals from the Guiana Shield forming two clades, one clustering the *C. albifrons* from Negro River and Orinoco, and the other clustering different subspecies of *C. olivaceus* (except *C. o. brunneus* of the Venezuela coast). Our results ratified this finding of Bobli et al., (2012), but our results also showed that the mixing of haplotype lineages or migrations are extremely more complex in the area of the Negro River and Orinoco than they detected.

E. Finally, there was a migration of *C. albifrons* haplotypes going east towards South America causing the emergence of the robust capuchins. As we did not study many *C. olivaceus* specimens or *C. kaapori* specimens, we don't know if the first ancestral robust capuchins haplotypes were generated directly from more ancestral *C. albifrons* haplotypes or if intermediate haplotypes were generated. For instance, ancestors of *C. kaapori* generated the ancestral haplotypes that generated the origins of the current *C. xanthosternos*, which is the first differentiated group of the robust capuchins. It's critical to study a good number of *C. kaapori* and *C. xanthosternos* individuals from a molecular perspective to detect shared, or very similar, haplotypes. If the ancestral haplotypes that generated the current *C. xantosthernos* were not themselves directly generated by ancestral *C. albifrons* or *C. kaapori* haplotypes then they should have also generated from *C. olivaceus* haplotypes. In Figure 5, we can observe that, from a karyotypic perspective, *C. olivaceus* is closely related to *C. apella* (*apella, cay* and *robustus*), with these species sharing the chromosomal inversion homologous to HSA20. Differentiation between *C. olivaceus* and *C. apella* is possible via a pericentric inversion in the association 14/15/14 and a Robertsonian rearrangement in the chromosomes homologous to HSA12 and HSA15b. Related to this hypothesis, Ford and Hobbs (1996) presented preliminary results that *C. olivaceus* is the gracile capuchin most similar to the robust capuchins in postcranial morphology. However, this morphological similarity does not necessarily show phylogenetic relationships as we discuss in different parts of this work. Also, von Dornum and Ruvolo (1999) showed at the nuclear *G6PD* locus that *C. olivaceus* was more related with *C. apella* than with the clade of *C. albifrons*-*C. capucinus*. Independent of these three hypotheses (the ancestors of *C. xantosternos* were derived from the ancestors of the current *C. albifrons, C. kaapori* or *C. olivaceus*), authors have shown that the Amazon and the Atlantic forests were connected for three million years (6 MYA until 3 MYA) allowing the migration of the gracile capuchins towards the Atlantic forests. The palynological studies of Oliveira et al., (1999) showed this fact. Schneider and Sampaio (2015) claimed that the split between the Amazon and the Atlantic *Callicebus* (*C. moloch* and *C. personatus*, respectively) and the divergence between the howler monkey species

(*Alouatta seniculus* and *A. fusca*) occurred during this period. Similarly, Nascimento et al., (2008) showed different populations of red-handed howler monkeys (*Alouatta belzebul*) from both forests. This suggests that two morphoclimatic domains with predominantly open vegetation biomes (Caatinga and Cerrado) split these populations around 3 MYA (Nascimento et al., 2008).

There is an intriguing question not revealed by previous studies on this topic (the existence of *C. albifrons* haplotypes within the robust capuchins cluster). There is a Western Amazon *C. albifrons* haplotype that is the sister clade of all the *C. apella* haplotypes. Also, there are five Western Amazon individuals of *C. albifrons* (Napo River area in Ecuador and Peru) mixed with the individuals of *C. a. macrocephalus* (also Western Amazon). Additionally, there are four Western Amazon individuals of *C. albifrons* (three from the Napo River area in Ecuador and one from the Colombian Amazon), two Orinoco *C. albifrons* individuals and the unique specimen analyzed of *C. a. malitosus* mixed with all the individuals of the *C. a. fatuellus* (Colombian Amazon and Orinoco). Different hypotheses have come forward from these results.

The first one is that this represents a case of incomplete lineage sorting. This phenomenon is due to that some lineages may occur in more than one taxon due to failure of two or more lineages in a population to coalesce in the ancestral population of each species (Ballard and Whitlock, 2004; Degnan and Rosenberg, 2009). Indeed, Nascimento et al., (2015) indicated polyphyletic arrangements for several capuchin taxa, suggesting that incomplete lineage sorting has occurred. If so, this could fundamentally support that the oldest gracile capuchin genetic diversity originated the current robust capuchin gene diversity. Furthermore, this process is relatively recent (Ballard and Whitlock, 2004; if not, incomplete lineage sorting would have disappeared), which is a relevant point against *Sapajus* as a different genus from *Cebus*. Taking this into account, different Western *C. albifrons* directly contributed to the generation of the robust capuchin haplotypes. This does not necessarily mean that *C. olivaceus* or *C. kaapori* haplotypes contributed to this fact (only an intensive genetics sampling of the robust capuchins in the Brazilian Atlantic forest could reveal this). Related with this, and if we take only into account the phylogenetic trees, these Western Amazon *C. albifrons* haplotypes (especially those from the Ecuadorian and the Peruvian Napo area) could be in the origin of the capuchins. However, the MJN analysis showed that these Western Amazon *C. albifrons* haplotypes were derived for other more ancestral gracile capuchin haplotypes as those from the Orinoco and Negro River and *C. capucinus* haplogroup III. Networks are more appropriate for intraspecific or very related species phylogenies (as it is the current case) than tree algorithms because they explicitly allow for the coexistence of ancestral and descendant haplotypes, whereas trees treat all sequences as terminal taxa (Posada and Crandall, 2001).

A second possibility is that after the robust capuchins returned to the Amazon (Lynch-Alfaro et al., 2012a), they experienced historic introgression with individuals of *C. albifrons*. In this case, all the introgression events detected should be between *C. apella* females and *C. albifrons* males. The descended hybrids bred over generations with *C. albifrons* because no *C. albifrons* individuals presented any morphological character of *C. apella*. In favor of this hypothesis we note the clade of *C. a. macrocephalus* which included *C. albifrons* from the most western area of the Amazon where both species sympatrically live (Ecuador). There were also two *C. albifrons*, within the *C. a. fatuellus* clade, also from the Colombian Eastern

Llanos where both species live sympatrically. If this second hypothesis is true, we would also expect to detect robust capuchin individuals within the main gracile capuchin clade (robust capuchin males x gracile capuchin females). However, we did not detect any such case. Thus, we are inclined to consider the first hypothesis as the most probably of the two.

The fact that unique *C. a. malitosus* individuals were enclosed in the *C. apella fatuellus* clade together with one Colombian Amazon *C. albifrons* could be interpreted as supporting the two previous hypotheses. If the first hypothesis was valid, then, *C. a. malitosus* could represent a sixth migration from the Western Amazon's *C. albifrons* array to what is currently Northern Colombia. This is different from the other migrations which generated Northern Colombia's *C. albifrons* populations. If the second hypothesis is assured, then *C. a. malitosus* could be interpreted as a linage formed by the hybridization of *C. albifrons* and *C. apella* in the Orinoco (Colombian-Venezuelan Llanos). Later, it migrated to the most northern Colombian areas. The very dark pelage of this taxon could support this last hypothesis.

Before discussing the evolution of the robust capuchins, we will comment about the most recent splits in the Central and Northern Colombian *C. albifrons*. The main splits of the Vaupes *C. albifrons* population, *adustus-leucocephalus*, *versicolor*, *cesarae* and *pleei* populations, which have traditionally been considered different subspecies, occurred between 0.4 and 1.1 MYA, during the Pleistocene. The mitochondrial diversification within some of these populations was estimated to have occurred around 0.8-0.13 MYA also during the Pleistocene. The Pleistocene is a fundamental epoch for the proliferation of haplotypes for *Cebus* and many other species. Shackleton and Opdyke (1973) and Bowen (1978) showed that the Pleistocene is characterized by glacial and inter-glacial cycles of around 100,000 Y due to the eccentricity of the Earth's orbit, with around 90,000 years of cold temperatures (glacial) and 10,000 years of warm temperatures (inter-glacial). Hays et al., (1976) also demonstrated that changing the Earth's axis from 22.1 ° to 24.5°, every 41,000 years, and its precession over 22,000 years have some minor influence on the glacial and inter-glacial Pleistocene cycles. Mercer (1984) detected glaciations in the Argentinian Patagonia since 3.5 MYA. Caviedes and Paskoff (1975) and Laugenie (1982) detected, at least, three large glaciations in the last 2 MY in Chile. Clapperton (1981) detected glacial deposits around 3.27 MYA near to La Paz (Bolivia). On the other hand, Bowen (1978) detected at least seven glacial and inter-glacial cycles with glaciations in the last 700,000 Y in the Britain Islands. In Colombia, Hooghiemstra (1984), analyzed palynological and lacustrine sediments and determined 27 large climatic cycles with a periodicity of 100,000 years, although there are only proofs, for Colombia, of the last large glaciation (Van der Hammen et al., 1983; Helmens, 1988). These alternations of cold-dry and warm-humid cycles suggest the possible existence of Plio-Pleistocene refugia following the hypothesis of Haffer (1969, 1997, 2008), provoking population contractions and expansions, which, in turn, could enable the apparition of multiple lineages of gracile capuchins in the Northern Amazon and Northern Colombia. This has had profound consequences, not only in the capuchins, but also in most of the South American fauna (Costa, 2003; Diniz-Filho et al., 2002; Hawkins and Diniz-Filho, 2006; Melo et al., 2009).

The Evolution of the Robust Capuchins: Their Origins in the Atlantic Forests to Again Return to the Amazon

It's clear that *C. xanthosternos* is the current robust capuchin most closely related to the original robust capuchins which derived from the gracile capuchins. Our results agree

extremely well with Nascimiento et al., (2015) and Ruiz-García et al., (2012b), who also detected *C. xanthosternos* as the first divergent robust capuchin clade. These results also are against the point of view of Lynch-Alfaro et al., (2012a), who consider that *C. nigritus cucullatus* to be the basal divergent taxon of the robust capuchins. Unfortunately, they came to this conclusion because they discarded a divergent sequence of *C. xanthosternos* (sample 49) because it was downloaded from GenBank of unknown provenance, and they considered a possible captive hybrid or an error resulting from contamination. With the MJN procedure, our split between the ancestral *C. albifrons* haplotype and *C. xanthosternos* was around 3.3 MYA during the last phase of the Pliocene. In the case of *C. xanthosternos*, the striking phenotypic differences of this taxon as described by Rylands et al., (2005), and being restricted to a determined geographical area, agree quite well with the fact that it should be the first divergent species within the robust clade. Seuánez et al., (1986) also determined some differences at the chromosomal level between *C. xanthosternos* and other lineages of *C. apella*. *C. flavia* (also known as *C. queirozi*; Mendes et al., 2006) is another first robust species candidate to diverge. It would be interesting to analyze it molecularly, since it is a new species of the robust capuchin complex and has been recently rediscovered (Oliveira and Langguth, 2006) from the Pernambuco endemism center (Brazilian states of Alagoas, Pernambuco, Paraíba and Rio Grande do Norte – north of the Sao Francisco River). However, we suggest *C. xanthosternos* as the best candidate of the current live robust capuchin forms to be most closely related with the original *C. capucinus*-*C. albifrons* group. Although *C. xanthosternos* belongs to the tufted robust group, it is an exception because it lacks tufts. Also, with age, some *C. albifrons* females (untufted capuchins) tend to develop a certain bushiness on the face and head, which in some cases includes tufts (Hill, 1960; Napier and Napier, 1967). This should be a phenotype link between both gracile and robust capuchin forms.

In a parallel study (Ruiz-García et al., 2016a), a Bayesian analysis revealed a temporal divergence between the gracile capuchin clade and the robust + gracile capuchin clade of around 5.52 MYA. The initial diversification in the first clade occurred around 5 MYA. This was followed by the initial diversification in the second clade around 3.68 MYA which split of the ancestors of *C. xanthosternos*. These temporal splits happened during the last phase of the Miocene and basically during the Pliocene. Casado et al., (2010) estimated the split between these two clades at 4.2 MYA, whilst they estimated the split between *C. a. apella* and *C. a. cay* to have occurred around 2.6 MYA (with a Bayesian procedure we obtained a split of 2.42 MYA, but with the MJN the split was around 0.3 MYA). This work together with those from Casado et al., (2010) and Ruiz-García et al., (2016a) detected the split between the gracile and the robust forms of *Cebus* to have occurred during the Pliocene. Lynch-Alfaro et al., (2012a) estimated an older temporal split between both forms (6.3 MYA; 95% highest posterior density: 4.1–9.4 MYA), to have happened during the Miocene. Nascimento et al., (2015) speculated that the Atlantic Forest was the original place of capuchin ancestors, in agreement with the hypothesis of the African origin of Neotropical primates (Schrago and Russo, 2003). Those capuchin ancestors reached the Atlantic coast of South America and subsequently dispersed to internal regions of this continent. However, this speculation is highly questionable because in the best cases the estimated split between both gracile and robust capuchins occurred 6-7 MYA. Compare this to the first recovered Neotropical primate fossil (*Branisella boliviensis*) dated to 27 MYA (Feagle, 2002; Takai et al., 2000) and the split between the South American and African Primates estimated at 35

MYA (Harrison, 1987) to 43.9 MYA (Hodgson et al., 2009). Thus, that the ancestral Neotropical primates reached the current Atlantic South American coasts is irrelevant to the split of the ancestral capuchin forms. The other alternative speculation of Nascimento et al., (2015) is more probable. They claimed that the ancestral robust capuchins may have originated in the western or central Amazon Forest and expanded their range to the Atlantic Forest, leading to a subsequent and complete speciation in the Amazon Forest. This also led to eliminating the basal robust capuchin form from this location. Then, *Cebus xanthosternos* represented a remnant distribution of a more widespread basal lineage. As we have commented, this is exactly what our results support. Another interesting difference between this work and that the Lynch-Alfaro et al., (2012a) is that they estimated the robust crown group age, to be around 2.7 MY. This is more than a half a million years older than the gracile crown group (2.1 MY). Additionally, they detected this first split of the gracile capuchin radiation between Amazon *Cebus* and Venezuelan Coastal/Central American *Cebus*. Clearly our data are more robust (includes many more individuals and localities and a higher number of genes sequenced) than the work of Lynch-Alfaro et al., (2012a). We also detected more ancestral *C. capucinus-C. albifrons* haplotypes which show that several hypotheses of Lynch-Alfaro et al., (2012a) could be refuted or, at least, to be transformed as we show below.

Indeed, Lynch-Alfaro et al., (2012a) argued that the temporal split of around 6-7 MYA for both gracile and robust capuchins is similar to that between the genera *Lagothrix* and *Brachyteles* (8.0 MYA, Barroso et al., 1997; 9.62 MYA, Meireles et al., 1999; 10.5 MYA, Schrago, 2007). Per se, these temporal comparisons are not very useful to distinguish two different genera in the capuchin monkeys. First, the value of the temporal split between *Lagothrix* and *Brachyteles* is considerably higher than the temporal split for the two clades of capuchin monkeys. This is true if it's compared with the estimate of Lynch-Alfaro et al., (2012a), but especially with our estimate of around 3-4 MYA. Second, several temporal splits between species within genera (and no one has claimed to divide these genera) are similar to the temporal split between the two capuchin clades. For example, consider the cases of *Ateles geoffroyi* and *A. paniscus* (4.36 MYA; Meireles et al., 1999) or *Callicebus torquatus* and *C. moloch* (5.5 MYA; Schneider et al., 1993). Third, they even argued that some genera of Neotropical primates are older than the split between *Sapajus* and *Cebus*. They used the example of *Alouatta*, where the split between the two main clades (cis- and trans-Andean) was around 6.8 MYA (Cortés-Ortiz et al., 2003), but arguing that for *Alouatta* there is no large morphological gap/distinction between species. There has been more continuous splitting and speciation through time in *Alouatta*. Furthermore, ecological niches are more similar across *Alouatta*, and the species are generally allopatric. However, in our view, Lynch-Alfaro et al., (2012b) failed to mention the main and more determinant point: the presence, or absence of reproductive cohesiveness, which implies the existence or not of pre and/or post reproductive isolation mechanisms. For instance, there would be more morphological homogeneity between the different *Alouatta* taxa than in the capuchin monkeys, but the extreme and conspicuous karyological differences among many *Alouatta* taxa show that there is reproductive isolation between many of them, whereas the reproductive cohesiveness is more certain in many capuchin taxa because there are not these conspicuous karyological differences.

After the divergence of the ancestors of *C. xanthosternos*, the next taxon to diverge was *C. nigritus* (although we did not analyze all the different morphotypes classified within *C. nigritus*). Mudry et al., (1991) showed that, although this taxon has relatively more similar

karyotypes to other robust capuchin taxa than *C. xanthosternos* did, it has different G banding for chromosome 11 compared to other *C. apella* populations. Additionally, the C banding in this group showed the loss of the large heterochromatic block on chromosome pair 11 present in other *C. apella* lineages. This taxon also showed eight small and eight large acrocentric chromosomes which disagrees with that determined for other robust capuchin taxa that have seven small and nine large acrocentric chromosomes (Freitas and Seuánez, 1982; Mantecón et al., 1984; Matayoshi et al., 1986). Furthermore, chromosomes 17 and 20 showed inconsistent heterochromatic blocks, which are absent in other *C. apella* taxa, as well as showing three of the small acrocentrics which correspond to pairs 22 and 23 and show a secondary constriction not present in other forms of *C. apella* . Indeed, Nieves et al., (2011) used a comparative genome hybridization analysis between *C. a. cay* and *C. nigritus* from Argentina where the DNA imbalances involved different genome regions. They were preferentially repetitive in *C. a. cay* or either coding or very disperse in *C. nigritus*. Their finding modified the accepted idea that the heterochromatin proportion is the only difference between *C. a. cay* and *C. nigritus*. These chromosomal characteristics of *C. nigritus* clearly contrasted with some of the chromosomal characteristics of *C. a. cay* found by Seuánez et al., (1983) and Matayoshi et al., (1986). Still, their geographical distribution could overlap in some areas of Eastern Paraguay and Northeastern Argentina. Indeed, Fantini et al., (2011) analyzed the genome of a female fertile hybrid between *C. libidinosus* (for us, *C. a. cay*) and *C. nigritus* using interspecies comparative genomic hybridization (iCGH). Although both taxa have a highly homologous karyotype and can interbreed, they detected different genome sizes supporting the species status for both taxa.

The remaining robust capuchin taxa (*cay, macrocephalus, fatuellus, apella* and one individual of *robustus*) showed very small genetic distances (average genetic distance of 0.4%) and a very recent diversification process (in the last 0.1-0.3 MYA). This rapid process of diversification was also detected by Lynch-Alfaro et al., (2012a), who estimated that the invasion of the robust capuchins from the Atlantic forest into the Cerrado and the Amazon occurred around 0.4 MYA. Also, Ruiz-García et al., (2012b), detected the split of all the *apella* populations from *C. xanthosternos* and *C. nigritus* to have occurred around 0.3 MYA. Indeed, all *apella* taxa showed very similar karyotype composition. Nieves et al., (2011) showed chromosome 11 to be the largest acrocentric pair. This chromosome has a heterochromatic block of 75% for the q arm. It is shared by all the robust capuchins, with the exception of *C. xanthosternos* and *C. nigritus*. Amaral et al., (2008) showed that *C. a. cay* presented a very similar karyotype to that of *C. a. robustus*, both having the same 12 synteny blocks with reference to humans and 18 identical conserved segments with regard to *Saguinus oedipus*. Additionally, the cladogram of Amaral et al., (2008) supported the monophylia of *apella, cay* and *robustus*. They shared two synapomorphic traits, the association 14/15/14, resulting in a submetacentric chromosome, and a pericentric inversion that corresponds to the HSA8b probe.

All of these taxa show a migration of the robust clade starting from the Brazilian Atlantic coast and going towards the west—crossing the dry areas of Southern Brazil, Paraguay and Northern Argentina to arrive to the Amazon of Bolivia, Peru, Ecuador, Colombia, Brazil, Venezuela and the area of Guianas. This confirms the point of view of Lynch-Alfaro et al., (2012a) that the robust clade migrated towards the Amazon from the Brazilian Atlantic forest in a very rapid and explosive radiation. This fact explains why the climatic variation seems to have influenced skull shape in robust capuchins to a larger extent than in gracile capuchins.

The main reason for this might be that the gracile capuchin ancestor lived in the Amazon rain Forest, which has existed for over 50 million years, ever since the Eocene (Hoorn et al., 2010). It is a comparatively more stable biome than others in the Neotropics during the Pleistocene (Mayle, 2004). This agrees quite well with the idea that robust capuchins experienced far more habitat fragmentation than the Amazon gracile capuchins during the Pleistocene. Therefore, this dynamic of strong habitat change and fragmentation outside of the Amazon may have spurred more morphological changes in the robust capuchins than in the gracile ones. Related with this, Casado et al., (2010) showed that *C. cay* populations suffered a recent demographic expansion during the Pleistocene, a period of repeated glaciation events leading to drastic changes in the vegetation composition of different biomes. This extreme explosive radiation generated many morphotypes highly adapted to the extremely different environments where these robust capuchins colonized (from the dry environment of the Paraguayan Chaco to the Amazon rain forest). But, molecularly and karyologically, these populations are extremely similar and, most likely, no reproductive barriers have been generated. The diversification within *cay*, *macrocephalus*, *apella* and *fatuellus* occurred in the last phase of the Pleistocene. This period had extreme climate changes. From 128,000 YA to 116,000 YA, there was an inter-glacial cycle. Since then, the last glacial period began and, for instance, in Colombia, the glaciers began to form around 70,000 YA (Wijmstra and Van der Hammen, 1974). Van der Hammen (1985) showed that the large glacier extensions were around 35,000 YA in this country. Later some reduction occurred and then about 25,000 YA the glaciers again extended. Although it was very cold, there was a reduction of the ice in Colombia from 21,000 to 14,000 YA. In contrast, in the Northern Hemisphere the maximum extension of the glaciers occurred around 18,000 YA. Also, the humid conditions in the area of what would become Colombia were insufficient for the advancement of the glaciers. For the last glacial phase, the temperature was 7 C° less than what it is today for the savannah of Bogota (Van der Hammen, 1985). Thus, these rapid and heavy climatic changes could influence the rapid morphological changes in *C. apella* but with very slight molecular and karyological changes without generating reproductive isolation mechanisms.

Lynch-Alfaro et al., (2012a) concluded that "Reinvasion of the Amazon" was the most feasible of the three hypotheses. They claimed that their data strongly supported the predictions of this hypothesis and give no support to the other hypotheses (out of the Amazon or Atlantic versus Amazon). Following these authors, their data upheld all three predictions of the first hypothesis: 1-the initial divergence in capuchin monkeys was between the robust Atlantic Forest capuchins and the gracile Amazon capuchins. They suggested that this process was initiated from vicariance caused by the establishment of the Amazon River around 7 MYA (Hoorn et al., 2010). The ancestral *Cebus* populations were restricted to the Guiana Shield, whereas the ancestral *Sapajus* populations were restricted to the Brazilian Shield. 2- The robust Amazon capuchins formed a recently evolved subclade nested within the Atlantic Forest robust clade. 3- Robust Amazon capuchins dispersed at a significantly higher rate than either gracile or Atlantic robust capuchins. They concluded that the current wide-ranging sympatry of *Cebus* and *Sapajus* across much of the Amazon is best explained by a Pliocene-Pleistocene diversification (2 MYA) of gracile capuchins in the Amazon. It is also explained by a much more recent expansion (0.7 MYA) of the Atlantic Forest *S. cay* into the southern Cerrado. This was followed by a single explosive Pleistocene invasion (0.4 MYA) of *S.*

apella/macrocephalus across the Amazon, where the robust capuchins are now sympatric with, or have displaced, gracile capuchins across a large portion of their range.

The current results clearly confirmed the second and third predictions of the "Reinvasion of the Amazon" hypothesis of Lynch-Alfaro et al., (2012a), but not the first prediction. Clearly, the mitochondrial evolution of the gracile capuchins started before the mitochondrial evolution of the robust capuchins. The robust capuchins are derived from some Amazon *C. albifrons* lineages and this is especially observable in the MNJ analysis. Unfortunately, this fact was never taken into account by Lynch-Alfaro et al., (2012a). Really, the evolution of the robust capuchins is nested within the evolution of the gracile capuchins. Therefore, the first prediction of the "Out of the Amazon" hypothesis (Amazon Basin is the ancestral origin of the capuchin monkeys), or a modification of it, is true. The original capuchin monkeys were gracile and they appeared north of the Amazon River (in the area of the Negro River/Orinoco, or in the northern Pacific area of current Colombia) around 1.5-2 MYA before the robust capuchins appeared in the Atlantic forest area. Later, the second and third predictions of the "Reinvasion of the Amazon" hypothesis of Lynch-Alfaro et al., (2012a) occurred. This new modified hypothesis is called by us the "North Amazon gracile origin, Eastern expansion and subsequent reinvasion of the Amazon."

The Necessity of a New Systematics for the *Cebus* Genus

We believe that the existence of different definitions of species is conceptually interesting and practically useful. However, there are situations where some species definitions are clearly more acceptable than others. For example, a researcher could be studying a sexual organism and only has limited molecular data, not related to reproductive incompatibility. Perhaps the researcher has some morphological or morphometric data too (not related to sexual characters and thus not considering reproductive isolation). If he/she has no any other information (relevant karyological differences, existence of pre and/or post-zygote reproductive barriers of whatever nature, clear geographic or ecological barriers which might cause allopatric speciation, etc, etc), from his/her desk in an office or in a laboratory, we think that it's licit to apply the PSC. Nevertheless, if a researcher could obtain (or there are previous studies) some results on the existence of direct, or indirect, reproductive data, the utilization of the BSC is clearly a preferable option over the PSC. In the last few decades, many primatologists, systematics and researchers of other biological sciences have "forgotten" the extreme importance of reproductive data in current sexual organisms, which is directly related with an intuitive idea of what a species is (Ayala, 1994a, b). We recommend to use the BSC over the PSC when direct and indirect reproductive data are presented. Herein, we will apply the BSC to the question of the systematics of *Cebus.*

Different *C. capucinus* haplogroups evolved in parallel from different *C. albifrons* groups (and/or vice versa) in the same areas of the Pacific and Northern Colombia, as well as in Central America. There were environmental selection pressures affecting the external morphology of these different lineages. They were helped by an intense gene flow among these original different groups. This hypothesis could very well be possible because in our species (*Homo sapiens*), this has been clearly documented. The action of natural selection on skin pigmentation reveals itself in several ways. For instance, note the relationship among UV radiation, vitamin D and skin melanin content (Neer, 1975). The geographical areas with high

incidence of UV radiation contain human populations with dark or very dark skin color. In this way the incidence of cancer skin is considerably lower (and reproductive fitness is higher). There is a higher protection of folic acid and vitamin D amount is enough to build bones with adequate levels of calcium. When humans left Africa and they colonized northern areas (Northern Europe and Northern Asia), the individuals with lighter skin were positively selected because they could more readily absorb small amounts of UV radiation in these latitudes and, therefore, form bones not suffering from rickets (Neer, 1975).

It's easy to observe that European and northern Asians have lighter skin. This has been independently achieved by convergent evolution. In the Europeans, genes were positively selected to lighten the skin. There are around 30 different alleles in Europeans and only a fixed allele found in Africans at the *MC1R* locus and one allele at the *SLC24A5* locus. Those selected in the Europeans were different to those selected in Northern Asians (allele *EDAR-V370A*, which also affects other characters such as the hair shape) (Fujimoto et al., 2008; Kamberov et al., 2013; Lamason et al., 2005; Parra, 2007). Thus similar environmental conditions could activate different gene variability to produce similar phenotypes (convergent evolution). Similar convergent effects on color, sizes and morphotypes could have occurred in many other Primates. Therefore, if this hypothesis is supported, the morphological similarity among different groups is the product of convergent natural selection. This would indicate that the traditional color pelage characteristics relied on by primatologists to carry out their systematic classification of many primate species should be not used. They are not good characters for phylogenetic tasks. Another probable hypothesis is that the original *C. capucinus* lineage (Colombian III haplogroup) was that which originally populated the entire geographical area of Northern Colombia. Then, at least, three *C. albifrons*' waves arrived and mixed without any problem with the *C. capucinus* residents (the group of Vaupes *C. a. albifrons* lineage-*C. a. versicolor*-*C. a. cesarae*-*C. a. pleei*; some direct Amazon haplotypes; and the *C. a. adustus*-*leucocephalus* group) generating the other detected *C. capucinus* haplogroups. Our results should reflect the introgression from males of *C. capucinus* to females of *C. albifrons.* Consider the hybrid we analyzed coming from the Bolivar Department. The specimen was physically more related to *C. capucinus* but it had the mitochondrial DNA of the *versicolor-cesarae-pleei* clade. In a second example, we found a different hybrid individual coming from the Sucre Department. It should represent a case of *C. a. versicolor*'s male introgression into Colombian III *C. capucinus* haplogroup females. The specimen was more physically related to *C. capucinus* but it had recognizable *C. albifrons* characteristics and mitochondrial DNA of the Colombian III *C. capucinus* haplogroup. Probably, the hybrids basically inherited dominant pelage characteristics (black versus clear colors) from *C. capucinus*. No matter which of the two hypotheses there were no reproductive barriers. As we previously commented, the karyotypes of *C. capucinus* and *C. albifrons* are very similar. Additionally, we showed in Ruiz-García and Castillo (2016), that these different *C. capucinus* haplogroups were highly affected by gene flow when nuclear DNA microsatellites were analyzed. Even Ruiz-García et al., (2016a) demonstrated that microsatellites results did not discriminate between *C. capucinus* and some *C. albifrons* groups. Thus, cohesive reproductive integrity is demonstrable among all of these lineages as we showed with the two hybrid exemplars directly sampled in the wild. Previously, other authors (Hernández-Camacho and Cooper, 1976; Defler, 2010) claimed the existence of hybrids between these two gracile capuchin monkey taxa.

Some hybrid animals were obtained in the Barranquilla market decades ago. Their reported origin was the Middle San Jorge Valley. They had intermediate features including a high dark crown, a white bald area on the forehead, and lighter fur on the outside of the arms and shoulders (all the features suggesting the predominant pattern of *C. capucinus*). Very likely, in the Magangue market, there were some hybrid individuals from the Lower Cauca River showing similar characteristics. The exception to this would be the lack of a very high, dark coloration. Additionally, many social conventions such as hand sniffing, sucking body parts, and inserting fingers into another individual's mouth have been described in *C. capucinus* as well as in *C. albifrons* (Defler, 2010), but not in *C. apella*. The description of vocalizations for *C. albifrons* by Defler (1979) matches closely to that of *C. capucinus*. In contrast, Di Bitetti (2002) described several quite different vocalizations for *C. nigritus*. Indeed, Hernández-Camacho and Cooper (1976) hinted that both, *C. capucinus* and *C. albifrons*, should be conspecifics.

For all of these reasons, we claim that all the gracile capuchins we studied conformed a unique species or super-species: *C. capucinus* because it is the oldest of the names for this taxon (*C. capucinus*, Linnaeus in 1758; *C. albifrons*, Humboldt in 1812; *C. olivaceus*, Schomburgk in 1848). We only analyzed one sample of *C. olivaceus* being placed within the Amazon *C. albifrons*'s haplotypes. The results of Bobli et al., (2012; see Figure 1), showed several samples of *C. olivaceus* (*C. o. castaneus, C. o. apiculatus, C. o. nigrivittatus* and *C. o. olivaceus*) more related to haplotypes of *C. a. albifrons, C. a. unicolor* or *C. a. yuracus* than to *C. o. brunneus*. The last one was more related to *C. albifrons* taxa such as *C. a. adustus* and *C. a. leucocephalus*. This also indicates that *C. olivaceus* should be enclosed within *C. capucinus*. However, our results could not be applied to *C. kaapori*, because we did not study any samples of that taxon. Thus, additional molecular analyses should be carried out to determine if *C. kaapori* needs be included within *C. capucinus* or if it should be considered an individual species. Following our molecular results, we cannot agree with some claims of Bobli et al., (2012). As we previously demonstrated (Ruiz-García et al., 2010, 2012a), they concluded that there is no molecular evidence for subspecific distinction of *C. c. limitaneus* in Guatemala and Honduras or *C. c. imitator* in Costa Rica. However, they concluded that the Central America *C. capucinus* should be differentiated as a new species, *C. imitator*. However, as we commented above, the new mitochondrial data of Ruiz-García and Castillo (2016) showed that haplotypes of the Colombian II haplogroup and Central American haplogroup were mixed in animals within the same troops. There were with no morphological differences in central and northern Panama and the nuclear DNA microsatellite results showed limited differences among Colombian, Costa Rican and Guatemalan individuals of *C. capucinus*. Thus, it does not seem realistic to describe, as did Boubli et al., (2012), the Central America capuchins as a new species.

The systematics of the different groups of the traditional *C. albifrons* (if they are not integrated in the superspecies, *C. capucinus*) from northern Colombia and Venezuela and the central and western Amazon is complex. Following the current molecular results and trying to correlate them with the traditional nomenclature rules and using the previous names from Hershkovitz (1949), Hernández-Camacho and Cooper (1976), Defler and Hernández-Camacho (2002) and Defler (2010), we conclude the following: 1- *C. a. malitosus* (Elliot, 1909) should be maintained although more specimens must be sequenced to strictly determine this taxon; 2- All of the previous taxa named as *cesarae* (Hershkovitz, 1949), *pleei* (Hershkovitz, 1949), *versicolor* (Pucheran, 1845) and *albifrons* (Humboldt, 1812; herein

unicolor was enclosed within *albifrons* following Defler and Hernández-Camacho, 2002) were intermixed throughout different clades. Thus, we named all of them as *C. a. albifrons*; 3- *C. a. leucocephalus* (*leucocephalus* is an older name than *adustus*, Gray, 1865 vs. Hershkovitz, 1949, respectively) proved to be a monophyletic group and 4- In the Amazon, the exemplars "a priori" classified as *albifrons* (and *unicolor*), *cuscinus* (Thomas, 1901), *yuracus* (Hershkovitz, 1949) and unspecified subspecies (Hershkovitz, 1949) were intermixed. We name all of them as *C. a. albifrons*. The question of *C. a. aequatorialis* and *C. a. trinitatus* is pending upon the molecular analysis of more samples because our preliminary results did not satisfactory resolve the question. Thus, we tentatively claim the existence of three taxa within *C. albifrons*: *C. a. malitosus*, *C. a. leucocephalus* and *C. a. albifrons*. Nevertheless, we consider it prudent to consider a unique species, *C. capucinus* with different lineages intermixed. Or, consider geographical populations with certain main characteristics (ESUs, Moritz, 1994, or other unities that do not necessarily correspond with color coat characteristics), where the traditional typological view of systematics cannot be easily (and really not desirable) applied. In fact, as Fontdevila and Moya (2003) explained, evolutionary biologists should make an effort to rule out the application of a typological view. The gracile capuchin case should be similar to that of human beings. We don't apply the traditional systematic nomenclature rules for our own species. For all of these reasons, we discard typological views of Bobli et al., (2012), who analyzed a limited number of mitochondrial genes from 50 *Cebus* individuals and supported the existence of 12 species of gracile capuchins. As we previously commented many of the haplotypes of these alleged taxa are intermixed and these authors never took into account the reproductive cohesiveness of all these forms. This demonstrates how the PSC can obscure our understanding of evolutionary phenomena and the speciation process.

In the case of the robust species, with the current molecular data we have, three species should be defined, although some populations and morphotypes have not been not studied by us: *C. xanthosternos*, *C. nigritus* and *C. apella*. The considerations we used to define the first two species were explained above. Our considerations for the third species are as follows. Our molecular analysis included specimens classified "a priori" as different species by morphological characteristics and by geographical origins. Following Groves (2001, 2005), they were: *C. apella apella*, *C. a. fatuellus*, *C. a. macrocephalus*, *C. a. peruanus*, *C. libidinosus pallidus*, *C. l. paraguayanus*, *C. l. juruanus* and *C. nigritus robustus*. Following Silva Jr (2001) they were *C. apella*, *C. macrocephalus*, *C. libidinosus*, *C. cay* and *C. robustus*. Our molecular results showed that *C. a. macrocephalus*, *C. a. peruanus*, *C. libidinosus pallidus*, *C. l. juruanus* and *C. nigritus robustus* (following the nomenclature of Groves, 2001, 2005) and *C. macrocephalus*, *C. libidinosus* and *C. robustus* (following the nomenclature of Silva Jr, 2001) conformed a unique and extremely homogeneous molecular clade we named *macrocephalus*. Other well defined clusters were *cay*, *apella* and *fatuellus*. However, our results as well as those of Casado et al., (2010) and Ruiz-García et al., (2012b), support limited gene differentiation between these taxa. For instance, *macrocephalus-cay* showed 0.2% of genetic differentiation, meanwhile *macrocephalus-apella* showed 0.7% of genetic differentiation and *fatuellus-cay* also showed the same degree of genetic divergence (0.7%). These values of genetic differentiation are considerably lower than the values obtained for different species within other Neotropical primate genera. Some examples are those from the genetic differentiation between *Callicebus lugens* and *C. torquatus* (4.0%; Casado et al., 2007), between *C. nigrifrons* and *C. personatus* (6.0%; Bonvicino et al., 2003),

between *Alouatta pigra* and *A. palliata* (4.0%; Nascimento et al., 2005), between *Saguinus midas* and *S. niger* (3.4%; Cropp et al., 1999) or between *S. bicolor* and *S. martinsi* (4.1%; Cropp et al., 1999). Similarly, and due to the low level of genetic divergence, Casado et al., (2010) found a lack of definition in different phylogenetic trees for solving the relationship between *cay* and *apella.*

If we use the BSC, undoubtedly, these robust capuchin taxa should be considered subspecies of a single species, *C. apella*. Indeed, Ruiz-García et al., (2016b) showed that the DNA microsatellite differences between two "a priori" different species of robust capuchins (Groves 2001, 2005), *C. libidinosus pallidus* (Bolivia) and *C. macrocephalus* (Peru and Western Brazilian Amazon) are extremely limited. This agrees with the current mitochondrial data where both robust capuchin taxa are undifferentiated. The highly differentiated morphotypes of these *C. apella* taxa generated in the last 0.2-0.4 MYA were submitted to very different climatic and ecological environments (due to different natural selection pressures). The explosive radiation is similar to what occurred in our own species (*Homo sapiens*) endured. *Homo sapiens* left Africa approximately 0.2-0.1 MYA (Cavalli-Sforza, 1997; Goldstein et al., 1995; Horai et al., 1995; Tishkoff et al., 1996; Vigilant et al., 1991) and was also submitted to very different climatic and ecological environments. This generated the noteworthy phenotypical diversity which we can see in our species. However, no serious scientific claims have stated that the current and different human morphotypes are different species. The molecular differences within our species are extremely limited and the reproductive integrity is a fact (BSC). We propose that the same rules that we apply to ourselves will be applied to the other Primates.

Many authors are applying the PSC, in an indiscriminate way, to many different organismal taxa, including mammals and primates. Nevertheless, the large-scale application of this concept has originated many ''crazy'' new species that are clearly not sustained by a critical analysis. This was named by Isaac et al., (2004) as "taxonomic inflation." Zachos et al., (2013) suggests that some of the proposed new mammal species are completely unjustifiable (new species of tigers as *Panthera sumatrae* and *P. sondaica*, Cracraft et al., 1998; Mazak and Groves, 2006; 12 new species of red deer, *Cervus elaphus*, Groves and Grubb, 2011; 11 new species of the small rocky antelope, *Oreotragus oreotragus* or new species of hares, *Lepus*, Palacios et al., 2008).

We agree with Nascimiento et al., (2015) that many cases of paraphyletic or polyphyletic arrangements can be explained by incomplete lineage sorting, as we previously argued. But this means that the split among the groups occurred recently as well (and/or) as the gene flow between these groups. This agrees quite well with the absence of reproductive barriers, which, in turn, is essential for the BSC. Also, we agree with the claim of Nascimiento et al., (2015) that any accurate phylogenetic reconstruction should include sequence data of multiple unlinked nuclear loci in several individuals of any given species. But we must recall that the discrimination power of the nuclear sequences is much lower than that of the mitochondrial DNA as we commented in the introduction for the infra-generic and specific levels. Collins and Dubach (2001) reconstructed the phylogeny of *Ateles* by means of two mitochondrial genes. However, they did not fully reconstruct it with a nuclear gene. In a similar fashion, Cortés-Ortiz et al., (2003) accurately reconstructed the phylogeny of *Alouatta* with mitochondrial genes but it was not possible with nuclear gene sequences.

Funk and Omland (2003) also suggested that polyphyletic and paraphyletic arrangements might be artifacts resulting from misidentified specimens and species limits, consequently

leading to inappropriate taxonomy (McKay and Zink, 2010). However, this was not the case of the current work because all the samples were obtained from wild individuals inhabiting their natural geographic areas.

What is clear is that we increased the number of individuals and geographical areas sampled and relied on a higher number of mitochondrial genes, than in previous studies. We showed that there is no reciprocal monophylia between gracile and robust capuchins and that the evolution of the gracile capuchins preceded that of the robust capuchins. Furthermore, the first was in the origin of the second, which was not detected in previous studies.

Finally, we agree with Zachos (2015) who stated that species are such fundamental units that they should not be introduced carelessly and that species description and splitting based on simple morphometric differences (even significant ones) or phylogenetic relationships derived from very limited molecular datasets should be strongly discouraged. They may serve to support conclusions derived from larger and more complete datasets, but are not enough on their own.

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